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Triggers and Mediators of Acute Exercise-Induced Cardioprotection

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Triggers and Mediators of Acute Exercise-Induced Cardioprotection

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2004

This Dissertation is dedicated to:

Melissa

"I wondered what would happen if I just climbed as if I were going to make it, with no uncertainty in my mind whatsoever."

-Royal Robbins

Acknowledgments

The Author would like to thank the following people for their help, contributions, and support to this effort:

FAMILY:

Melissa, Pat, Mac, Jay, Colin, Betty, and Carmac Sr. Taylor; Sydney and Jake Hammons; Mary Jane, John, and Terry Hall; Melanie, Dan, Jake, and Madeline Fowler.

COMMITTEE:

Joe Starnes, Roger Farrar, Jim Kehrer, Barrie Kitto, and Ken Diller.

FRIENDS:

Brennan, Jason, Matt, Brian, Marissa, Ding, Donovan, James, Chris, Mina, Patty, Jan, and Cindy.

FUNDING:

The American College of Sports Medicine Foundation Research Grant, The University of Texas Continuing Fellowship, The Donald D. Harrington Dissertation Fellowship, and The Lynn Wade McCraw Endowed Presidential Fellowship in Education.

Triggers and Mediators of Acute Exercise-Induced Cardioprotection

Publication No._____

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The University of Texas at Austin, 2004

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Introduction: Acute exercise, consisting of 1 to 3 bouts of 60 to 100 minutes, is capable of preconditioning the myocardium against ischemia-reperfusion (I/R) injury. We previously reported that elevated heat shock protein 70 (HSP70) is not required for this cardioprotective effect. The agent or agents that trigger and mediate this preconditioning effect are still not understood, but may involve the production of reactive oxygen species (ROS) during exercise as a trigger, and the production of nitric oxide (NO) by nitric oxide synthase (NOS) during I/R or uncoupling of mitochondrial respiration as mediators. The purpose of the present studies was to determine the role of these potential triggers and mediators in acute exercise-induced (AEI) cardioprotection.

Methods: Rats were randomly assigned to seven treatment groups: sedentary (SED);

two days of treadmill exercise at 20 m/min, 6-degree grade, for 60 minutes (RUN); sedentary and injected with 100 mg/kg N-(2-Mercaptopropionyl)glycine (MPG, a potent ROS scavenger) (SED/MPG); exercise and injected with MPG (RUN/MPG); sedentary then perfused with 100 μM Lω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, a competitive inhibitor of NOS) (SED/L-N); exercise then perfused with L-NAME (RUN/L-N); exercise in a 4°C environment (to prevent HSP70 increase) then perfused with L-NAME (CRUN/L-N). Twenty-four hours following the second exercise bout, or MPG injection, isolated-perfused working hearts were subjected to 22.5 minutes of global ischemia followed by 30 minutes of normoxic reperfusion. Portions of left ventricle were analyzed for several putative mediators of exercise-induced cardioprotection including: HSP70, inducible (iNOS) or endothelial (eNOS) NOS, manganese superoxide dismutase (MnSOD), and catalase. **Results:** All exercise groups displayed improved recovery of cardiac function vs. sedentary groups, which was not inhibited by MPG injection or L-NAME perfusion. Also, all exercise groups had improved efficiency post-ischemia, suggesting that uncoupling does not mediate AEI cardioprotection. The only increases in protein expression were: HSP70 in RUN, RUN/MPG, and RUN/L-N; eNOS in CRUN/L-N; and catalase in SED/MPG and RUN/MPG. **Conclusions:** AEI cardioprotection appears to not be triggered by ROS production, is mediated by changes independent of eNOS or catalase expression and uncoupling, and can occur in the absence of increases in HSP70, MnSOD, and iNOS.

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Introduction

Several researchers have reported that chronic exercise training can improve the ability of the heart to recover pump function following a period of ischemia (25, 26, 28, 42, 61, 72, 81, 95, 103, 128, 152). It is now known that only a few bouts of exercise (acute exercise) are required to receive the cardioprotective benefits previously only attributed to chronic training programs (43, 49, 58, 65, 93, 98, 124, 145, 158, 172, 173). However, the mechanisms that provide this protection are not fully understood. In 1995, Locke et al. (98) suggested that the cardioprotection provided by three days of exercise was due to an exercise-induced increase in the myocardial concentration of heat shock protein 70 (HSP70), because it positively correlated with increased functional tolerance to ischemia-reperfusion (I/R) in a Langendorff heart perfusion model. To determine whether increased HSP70 is required for acute exercise-induced cardioprotection, we designed an experiment to prevent a rise in the concentration of HSP70 by exercising animals in the cold (158). Not only did we find improved cardioprotection against I/R injury 24 hours after exercise in the cold, we also found that enhanced protection was present after only a single bout of exercise. Our finding that elevated HSP70 is not required for the cardioprotection induced by acute exercise was subsequently confirmed by Hamilton et al. (58) and Lennon et al. (93). While these studies were able to dissociate HSP70 from acute exercise-induced cardioprotection, the mechanisms responsible for the protection were not addressed and are still not well understood.

A subsequent study carried out in our lab (157) provided some insight into other possible mechanisms underlying exercise-induced cardioprotection. Reactive oxygen species (ROS) are produced in the myocardium during exercise and I/R, and have been widely implicated in the damage associated with I/R injury (30, 167). Cardiac dysfunction due to ROS can be modeled by exposing an isolated-perfused heart to pro-oxidants such as hydrogen peroxide (H_2O_2) (143). We found that chronic exercise did not protect cardiac pump function in response to H_2O_2 exposure; however, the release of the cytosolic enzyme lactate dehydrogenase (LDH), an indicator of sarcolemma damage, was attenuated by exercise training. One of the most interesting, and relevant, findings of this study was that exercise enhanced the coronary flow (CF) response to H_2O_2, which was potentially due to an increase in nitric oxide synthase (NOS) following exercise training. When we paired this finding with our previous observation that CF was significantly elevated following I/R in the group that exercised in the cold (158), we made the following speculation: the mechanism by which acute exercise provides cardioprotection, independent of HSP70, could be related to a ROS-induced increase in CF through the action of nitric oxide (NO) produced by an increased concentration of NOS in the myocardium.

The observation, of a positive relationship between CF and increased cardioprotection marked the beginning of our investigation into the triggers (signal/s that cause adaptations within the myocardium) and the mediators (agent/s responsible for providing the direct protection) of cardioprotection. As previously mentioned, the production of NO during reperfusion may have a cardioprotective effect, which has

been characterized by numerous investigators (17); however, other changes in the myocardium may occur as a result of exercise that allow for redundant mechanisms of protection. ROS production during I/R appears to be a major contributing factor to myocardial damage and dysfunction and some investigators believe that suppression of the I/R-induced production of ROS could prevent the damage associated with I/R injury. One method proposed to attenuate the rise in ROS during I/R is an uncoupling of mitochondrial respiration (16, 132, 159). Additionally, increases in various anti-oxidants could also serve the same purpose of decreasing the concentration of ROS in the myocardium. Antioxidant enzymes have been observed to increase (43, 173) or not increase (98) with acute exercise, but do not appear to be involved with chronic exercise induced cardioprotection (61). Attenuating the rise in ROS during I/R would appear to have protective effects for the myocardium; however, their presence during exercise may be required to trigger cardioprotective changes (2, 77, 82, 156, 160, 179, 180). Similarly, the production of NO during exercise may be an essential trigger for related cardioprotective changes (1, 4).

The purpose of this study is to elucidate the mechanisms by which 2 days x 60 minutes of exercise triggers cardioprotective adaptations and to determine which agent(s) are responsible for mediating the cardioprotective response. Through the execution of the experiments outlined herein, an attempt will be made to answer four primary questions pertaining to the triggering and mediation of cardioprotection by acute exercise. First, is there a difference in cardiac efficiency (oxygen utilization relative to external work being performed) following acute exercise and if so, are any

differences due to increases in uncoupling protein 2 (UCP2)? Second, does an acute exercise-induced increase in ROS trigger the cardioprotective response? Third, does an acute exercise-induced increase in NO trigger the cardioprotective response? Finally, does NOS mediate exercise-induced cardioprotection against I/R injury and will inhibition of NO production during perfusion block cardioprotective changes? Through the information gained during the investigation of these objectives, a better understanding of acute exercise-induced cardioprotection will be gained, which will aid in the continuing development of clinical cardioprotective therapies.

Significance

Because cardiovascular disease is prevalent in America, actions must be taken in order to prevent deaths and to improve one's chances of surviving a heart attack. Many different approaches, including exercise, have been investigated in the process of addressing this problem. Until recently, the common belief was that in order to receive cardioprotective benefits, exercise must occur on a regular basis. This dosage of exercise is well above what many Americans regularly perform. Previous work has demonstrated that a single bout of exercise is capable of inducing protective changes in the myocardium against I/R injury, similar in magnitude to those found after months of regular exercise. A number of upstream cell-signaling events, including activation of protein kinase C epsilon (PKCε), appear to be essential for the development of cardioprotection; however, the specific signals that trigger this activation and the downstream agents that mediate cardioprotection are not fully understood. The findings of this study will help elucidate the link between acute exercise and the specific mechanisms that trigger and mediate the cardioprotective response to acute exercise. These findings will further our understanding of normal cardioprotective responses, so that future cardioprotective interventions and therapies may be developed.

Review of Related Literature

Background. It is well-documented that physical activity lowers the risk of cardiovascular disease and reduces deaths resulting from heart attacks (35, 91, 116); however, the mechanism(s) underlying the observed protection were not, and are still not, fully understood. One of the earliest studies to directly address the question of whether or not exercise induced changes in the heart muscle itself was carried out by Bowles et al. (25) who found that 11 weeks of treadmill exercise provided protection against I/R injury in an isolated-perfused rat heart. A common cause of damage to the myocardium in humans is ischemic injury due to temporary (total or partial) blockage of the coronary vasculature (122). The isolated-perfused heart model (diagrammed in Figure 1) is unique in that it allows for direct measurement of cardiac function, independent of any nervous, hormonal, or hemodynamic influences (119). The findings of Bowles et al. represented a huge leap in the understanding of exercise-induced modification of cardiovascular health, but the study provided little insight into the mechanisms of protection or the time course for the achievement of a cardioprotective effect. The American Heart Association now categorizes lack of physical activity as a risk factor for cardiovascular disease, which is the leading cause of death in the United States. Recent studies have demonstrated that as few as one day of exercise can provide a cardioprotective effect similar to that observed following several weeks of exercise. Exploration of the acute exercise-induced cardioprotection phenomenon will aid in the development of reasonably implemented exercise programs, and as the physiological

mechanisms responsible for protecting the myocardium are better understood, additional cardioprotective interventions may be developed.

Ischemia and Preconditioning. Cardiac dysfunction due to I/R injury is normally grouped into two distinct types of injury, myocardial stunning and the more severe and irreversible myocardial infarction caused by necrosis and/or apoptosis (21). The exact mechanism(s) by which myocardial stunning causes dysfunction is not known, but it has been proposed that increased ROS production, calcium overload, or a combination thereof may be responsible. The amount of cardiac function that is recovered following I/R will depend upon the degree of damage, which is directly related to the duration of ischemia. Although stunning is not as severe as necrosis of myocardial tissue, if the heart is not able to recover from a period of myocardial stunning, the duration of the ischemic insult will increase thereby further increasing myocardial damage. Therefore, protection against the specific phenomenon of myocardial stunning is a valid clinical objective.

Preconditioning is a phenomenon by which a stress applied to a tissue or organ initiates a change or changes, leading to protection against subsequent exposures to similar or different types of stress (18). Methods currently known to precondition the myocardium include: exercise (25, 98, 158), ischemic preconditioning (117), heat stress (39, 69), oxidative stress (57, 138), stretch (123), and certain pharmacological interventions (165). Although a majority of the research into the preconditioning phenomenon has been carried out using cell cultures and laboratory animals, there is significant clinical evidence, in humans, for a cardioprotective effect of a

preconditioning stimulus (15, 104, 108). The time frame over which preconditioning occurs can be divided into two distinct periods, the early phase and late phase, with each protecting by very different mechanisms. Early phase protection develops within minutes following a preconditioning stimulus, may last as long as 2 to 3 hours, and is primarily mediated through the post-translational modification of existing proteins. Late phase protection is mediated through *de novo* synthesis of protective proteins, develops within 12 to 48 hours, and may last as long as 9 days following a preconditioning stimulus. Some of these putative protective proteins synthesized include HSP70 and manganese superoxide dismutase (MnSOD). Another distinct difference between the two phases of protection is that late phase preconditioning can protect against both infarction and stunning, but early phase preconditioning only appears to protect solely against infarction (18). When considered together, all of the various aspects of myocardial damage and the numerous methods known to protect the myocardium constitute numerous potential investigative targets. Therefore, the current investigation is limited to the examination of only a limited number of the specific aspects of these phenomena including the late phase preconditioning of the myocardium against ischemia-induced myocardial stunning by acute exercise.

Acute Exercise. In 1995 a group of investigators found that exposure to a single, 15 minute, sub-lethal heat-stress or to as few as 3 days (but not a single day) of treadmill exercise could precondition against I/R injury in a Langendorff-perfused heart (98). Additionally, they found that improved post-ischemic recovery of mechanical function resulting from exercise or passive heat-stress was significantly correlated with

an increase in heat shock protein 70 (HSP70). This was an important finding, as no previous reports had demonstrated such a strong effect of only an acute exposure to exercise, but it was not clear if the observations of the investigators would be found when myocardial function was evaluated in the more sensitive and physiologically relevant isolated working heart. Subsequently, we performed a series of experiments to determine whether or not one or three days of exercise could provide similar protection in the isolated working heart (158), which led to some interesting findings. First, the findings of Locke et al. (98) were confirmed in the working heart model, as 3 days of treadmill exercise protected against I/R injury in the isolated working heart. Second, it was determined that one day of exercise could provide a similar degree of protection, which may have been due to the increased sensitivity of the working heart model, or to a slightly longer running time (60 vs. 100 minutes). Third, a similar degree of protection was provided when rats were run for one day in a cold (8°C) environmental chamber, thereby preventing a rise in core temperature (~41°C) and increase in HSP70 that normally accompanies treadmill exercise in a 20°C environment. Therefore, an increase in HSP70 expression was not required for improved cardioprotection. Finally, we observed that the coronary flow (CF) during reperfusion was elevated in the cold exercisers compared to all other groups. A potential explanation for this observation is that cold running resulted in increased production of myocardial NO, a potent vasodilator and possible mediator of protection against I/R injury (17). The agent or agents responsible for providing the protection in the preceding experiment and the signals that initiated the development of the cardioprotective phenotype are not

completely understood. The remaining sections of this review will explore the current knowledge of cardioprotection and I/R injury, and discuss a series of proteins and pathways that may be involved in exercise-induced cardioprotection.

Oxidative Stress. The flow of electrons through the electron transport chain is a necessary step in the aerobic production of ATP in mitochondria; however, a consequence of this process can be production of ROS including superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) (Figure 2). Peroxynitrite ($ONOO^-$), a reactive nitrogen species, is also produced during this process (Figure 2). One-electron reduction of O_2 appears to be the primary source of ROS in the cell; however, there is considerable debate as to where specifically in the electron transport chain the suspect electrons originate (Complex I, Complex II, or ubiquinone), and to the conditions under which they can be produced (state 3 vs. state 4) (see (161) and (92) for review). Additionally, it has been proposed that ROS production could originate from other sources in the rat myocardium including NADPH oxidase (169) and xanthine oxidase (31, 135), but the production of ROS from xanthine oxidase may be a rat model-specific phenomenon (31) as the enzyme does not appear to be present in rabbit (50) or human (51) hearts.

Experimental data from studies of intact tissues support the production of ROS during exercise (10, 121, 137) and I/R (9, 53); therefore, understanding the mechanisms capable of decreasing the damage due to ROS during I/R will be essential when describing the cardioprotective phenotype. It is important to note that the ROS produced during exercise may (96) or may not (10, 121, 137) damage the myocardium; however,

they may play an important role in the development of cardioprotection, which will be discussed below. Additionally, total abrogation of the production of ROS may not be an acceptable solution to preventing I/R injury as a limited presence during reperfusion may actually enhance recovery (82) and production during exercise may trigger a preconditioning response (36). Two strategies proposed for reducing the damage caused by ROS during I/R include: 1) reducing the amount of ROS produced or 2) increasing the amount of radical scavengers (including: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX)) available to dispose of ROS (Figure 2).

Mitochondrial Uncoupling. Electrons escaping from the electron transport chain have been implicated in the production of ROS resulting in oxidative/reductive stress. Studies performed on isolated mitochondria suggest that during state 4 respiration (No ADP, highly reduced electron carriers) ROS production is high while during state 3 respiration (ADP present, high rate of electron flux) ROS production is dramatically reduced (63). The addition of ADP allows for high rates of ATP production, and during this process the proton gradient across the inner mitochondrial membrane is partially dispersed. This physiological dispersion of the proton gradient, and resulting oxidation of the components of the electron transport chain, can also be accomplished through the addition of pharmacological uncouplers of mitochondrial respiration such as dinitrophenol (DNP). Cardiac efficiency is the measure of the amount of cardiac work (COxSP) divided by the amount of oxygen that the heart is consuming at any given time. If the assumption is made that the efficiency of force production from the hydrolysis of ATP is not affected during perfusion and that the amount of work that the

heart is performing does not change, any increases in the amount of oxygen being consumed would be due to a decreased efficiency of energy production. In fact, a small dose of DNP has been shown to decrease the production of ROS (16) and to protect against I/R injury in isolated-perfused hearts (111). It is also important to note that DNP decreases cardiac efficiency, before and after I/R in isolated-perfused hearts (16), which one would expect to observe with an uncoupling agent.

Uncoupling proteins. There is evidence for a physiological method of mitochondrial uncoupling in the myocardium through the action of a protein known as uncoupling protein 2 (UCP2) (22). Although an entire family of uncoupling proteins exists, UCP2 is the predominant isoform expressed in the heart (22). Furthermore, transfection of either UCP1 (14) or UCP2 (159) into cardiomyocytes resulted in protection against hypoxia and ROS induced damage. To date, no studies have evaluated the effects of acute exercise on UCP2 expression; however, one study found that chronic exercise decreases myocardial UCP2 mRNA expression (23). It would appear to be counterintuitive to investigate a protein which seems to be down-regulated with exercise, but the responses of the myocardium to chronic vs. acute exercise may differ substantially, and mRNA levels at a single time point may not accurately reflect protein expression (46, 114). It should also be noted that exposure to a cold environment increases UCP2 mRNA in the hearts of rats (24), which at first glance would appear to complicate the interpretation of any results from animals exercising in a cold environment. However, we have previously determined that increased heat production from running is sufficient to prevent hypothermia. The temperature of the

animals while running is an important variable in acute exercise-induced cardioprotection and will be discussed in following sections.

Antioxidants. Antioxidant enzymes are an essential part of the physiological defense mechanism (Figure 2) and over-expression of many of the predominant antioxidants, including: MnSOD (34), CuZnSOD (33), CAT (94), and GPX (140), has been demonstrated to protect the myocardium. However, regulation of their expression in response to a preconditioning stimulus is the subject of much debate. Although antioxidants clearly have protective effects and are constitutively expressed, their contribution to exercise-induced cardioprotection is questionable and they do not appear to mediate the late-phase protection provided by ischemic preconditioning (155). A study from our laboratory indicates that myocardial antioxidant enzymes are not elevated after adapting to a chronic exercise program and that exercise-induced protection against I/R injury associated with chronic exercise is not dependent upon an up-regulation of the expression of the predominant antioxidant enzymes (61). However, there is some evidence that antioxidant enzymes may be increased during the early phase of adaptation to a chronic exercise stress. For example, Somani et al. (144) reported increases in MnSOD, CAT, and GPX activity following a single exhaustive bout of exercise by previously sedentary rats. The findings of these authors are somewhat suspect, since the animals were sacrificed immediately following the exhaustive exercise bout at an intensity representing approximately 100% of VO_2max. If increases in enzyme activity represent an increase in protein content, this would require that all increases in protein expression occur in a time frame less than 100

minutes, which may not be possible due to the temporal constraints of transcription and translation. A potential explanation for the unusual findings of the authors can be found in a study by Yamashita et al. (173), who report that MnSOD specific activity is increased 30 minutes following a single bout of exercise, but that the concentration of the protein does not increase until 48 hours following cessation of exercise.

Additional studies from different groups of investigators further complicate our understanding of the effect of acute exercise-induced cardioprotection. Three separate studies from the same laboratory found that acute exercise may increase MnSOD and HSP70 only (43, 58), or alternatively, only CAT and HSP70 but not CuZnSOD or MnSOD (93). The increase in HSP70 and/or MnSOD are in agreement with other acute exercise studies (65, 98, 173), but increases in CAT with acute exercise have not been reported in other studies (98). Overall, a consensus has yet to be reached among investigators regarding the expression of MnSOD and CAT after exercise. This may be due to the method by which the enzymes are measured (activity vs. protein content (173)), the strain of the animal (Sprague Dawley (43, 58, 93, 98) vs. Wistar (173) vs. Fisher 344 (144)), gender of the animal (male (93, 98, 144, 173) vs. female (43, 58)), the number of days considered to be acute exercise (1 (144, 173), 2 (65), 3 (98), 8 (93), 9 (58), or 10 (43)), or age (145). To our knowledge, no studies have found CuZnSOD to be elevated after acute exercise and the only study to report an increase in GPX is the study by Somani et al. discussed above as having some experimental design problems (144). Therefore, due to the fact that CuZnSOD and GPX do not appear to change in response to acute exercise at any age (145) they are not evaluated in the present study.

The current study will attempt to clarify the myocardial response to acute exercise, but more importantly, any differential patterns of expression in response to the various experimental conditions may explain some of the differences reported in the literature.

Reactive Oxygen Species as a Trigger of Cardioprotection. ROS appear to play a role in the damage and dysfunction due to I/R, but their production during exercise may play an essential role in signaling the preconditioning of the myocardium (77, 173, 179). Initially, two groups of investigators found that the administration of a combination of antioxidants; including SOD, CAT, dimethylthiourea (DMTU), and N-(2-mercaptopropionyl)glycine (MPG), could block the late-phase preconditioning effect of ischemic preconditioning (149, 154). However, subsequent studies have reported that administration of MPG alone shortly before a preconditioning stimulus is sufficient to block the preconditioning effects of ischemic (156, 160), heat stress (2, 175), or ROS-donor exposure (153, 179) preconditioning.

Whether antioxidant administration prior to exercise will block exercise-induced cardioprotection is unclear, as the only two studies to evaluate this phenomenon reported conflicting results (59, 173). Hamilton et al. (59) reported that a diet high in antioxidants will improve protection against stunning in response to short I/R and that antioxidant diet plus acute exercise will further enhance recovery of mechanical function. In response to long I/R (potential myocardial necrosis/apoptosis), antioxidant diet plus exercise, antioxidant diet alone, and exercise alone, all resulted in similar infarcts compared to sedentary animals on a control diet; however, the combination of antioxidant diet and exercise did not produce better results than either individually.

Hamilton et al. also found that MnSOD was increased by exercise regardless of diet. These are the opposite of those reported by Yamashita et al. (173) who found that MPG administration plus exercise abrogated the increases in MnSOD and I/R tolerance (infarct size) that occur 48 hours after a single exercise bout without MPG.

There are a number of issues to be addressed in the study by Hamilton et al. that may be contributing to the differences reported. First, the lack of an abrogating effect, with respect to functional differences between the exercised vs. exercised with antioxidants, may be due to the low sensitivity and decreased control of experimental conditions in the in-vivo heart perfusion. This is apparent in that sedentary animals decreased function approximately 30% and exercised animals recovered greater than 100% following ischemia. The magnitude of the difference between the two exercising groups is very small, and any protective effects could be due solely to the antioxidant exposure. It is interesting to note that the expression of HSP70 in response to the exercise was blocked by the antioxidant diet in a manner similar to that observed when heat-stress preconditioning is blocked by MPG (175), and there is significant evidence for ROS-mediated mechanism of HSP70 expression(86, 120, 153). Second, prior to performing exercise the animals were fed the antioxidant diet for 6 weeks resulting in a chronic increase in the total antioxidant capacity of the heart, as opposed to being given a single dose of MPG, which is short acting and provides antioxidant protection during the exercise sessions only. Third, the animals were exercised for a total of 10 days, including the habituation period, which may indicate a pattern of chronic exercise adaptation rather than an acute adaptation, protecting via alternate mechanisms. Due to

the fact that Yamashita et al. (173) only evaluated 20 minutes of exercise-induced protection against long I/R injury and that the experimental design of the study by Hamilton et al. leaves some uncertainty, the adaptive response of the myocardium to acute exercise is still not apparent. Clearly, additional work will be required in order to determine the role of ROS in acute exercise-induced cardioprotection and how the various protective responses are triggered.

HSP70 expression and regulation. The increased synthesis of HSP70 in response to heat-stress, first observed in 1962 (130), has a widely recognized role in cellular protection (80), and has emerged as a primary mediator of cardioprotection due to preconditioning (80, 85, 89). The first study to demonstrate heat stress-induced protection from ischemia-induced damage, mediated by HSP70, was carried out by Currie et al. in 1988 (39). Subsequently, adenovirus transfection studies designed to over-express HSP70 in rat hearts indicated a direct role of the protein in protection against dysfunction and necrosis after an ischemic insult (150, 151). Studies from our laboratory suggest that increased body temperature is the primary signal for increased synthesis of HSP70 following either acute (158) or chronic (61) exercise. However, the minimal myocardial temperature required for signaling an increased HSP70 production is unclear as no studies to date have reported a direct measurement of myocardial temperature during exercise, most likely due to the invasive procedures involved with such a measurement. Rectal temperature is commonly used as an indicator of core temperature (29, 43, 54, 58, 61, 110, 134, 142, 158) and should provide a relatively accurate representation of the core temperature of the animal during exercise. The

minimal rectal temperature required for increased synthesis of HSP70 in response to exercise has been reported to be 39.9°C (110), however, previous studies have reported that animals running at similar intensities have much higher (~44°C vs. ~40°C) rectal temperatures (29, 134). The general consensus in the literature is that there is an absence of heat stress-mediated effects in whole organisms below 39°C (109), and the animals to be exercised in this study should be either well above or well below this threshold as indicated in previous studies (158).

In order to understand how acute exercise triggers cardioprotection, with or without HSP70, the regulation of HSP70 expression must be understood. Once the basic mechanisms controlling this process are understood, it will become clear that there is significant overlap and redundancy in the regulation of the entire cardioprotective response and that the regulation of cardioprotection is simply a balance between redundant mechanisms. The primary transcription factor responsible for controlling HSP70 is heat shock factor-1 (HSF1), and transcriptional control is regulated in part by PKCε (80, 89). PKCε is an important component of the cell-signaling mechanism of the myocardium (163) and is capable of phosphorylating a number of different proteins, including HSF1 (48, 107). The activation of HSF1 through phosphorylation facilitates the transcription of HSP70 in response to heat stress by promoting the trimerization HSF1, which can then bind to the heat shock element in the promoter region of the HSP gene to increase transcription (47). The activation of PKCε appears to be mediated by the production of both ROS and NO (5, 7, 171), and the over-expression of activated PKCε has a clear cardioprotective effect (6, 38). The information available regarding

acute exercise, which produces ROS and NO, suggests that a single bout of exercise activates PKCε and provides a cardioprotective effect (32, 172). Therefore any increases in HSP70 following acute exercise may be due to the trimerization of phosphorylated HSF1 and the cardioprotection provided by acute exercise in the cold could be mediated by the up-regulation of other cardioprotective targets also controlled by PKCε. As with many cellular regulation processes the expression of HSP70 can regulate its own expression through feedback inhibition of the activation of PKCε (48). Therefore, one would assume that if HSP70 expression is not increased in response to an acute stress (exercise), and that the heat-stimulated trimerization of HSF1 is the only absent stimuli, that other triggers of cardioprotection, including ROS and NO, could hyper-activate PKCε and increase other cardioprotective proteins, including NOS.

Nitric Oxide as a Trigger of Cardioprotection. Nitric oxide is claimed to have a dual role of triggering and mediating cardioprotection (17). There is strong evidence for a triggering effect of NO from studies reporting that NO donors are capable of inducing a cardioprotective effect (8, 64, 102, 127, 153), and that inhibitors of NOS are capable of blocking preconditioning stimuli of pharmacological agents (12, 62), ischemia (131), or heat-stress (74). The physiological source of NO that triggers preconditioning appears to be eNOS as the non isoform specific inhibitor Lω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) is capable of blocking a preconditioning response, whereas the iNOS-specific inhibitor aminoguanidine does not have the same abrogating effect (20). The preconditioning effect of NO may be mediated through PKCε, potentially requiring ROS for the formation of $ONOO^-$ as the stimulating agent (7, 153).

The expression of HSP70 may be linked to NO production as administration of exogenous NO donors can increase expression of HSP70 (101, 170) and inhibitors of NOS are capable of blocking heat stress-induced increases in HSP70 (78, 79, 100). Activation of PKCε may be required for the stimulation of increased HSP70 expression, but it may also regulate the transcriptional activity of iNOS (73). The preconditioning effects of pharmacological agents (181), ischemia (73, 166), and heat stress (1) all appear to trigger cardioprotection mediated by iNOS, and the cardioprotective effect can be blocked by either iNOS gene knockout (71) or NOS inhibitors (1, 27, 73). The only study to evaluate NOS expression following acute exercise reports that there is no increase in mRNA for any of the three isoforms (70), which does not resemble the pattern observed with heat-stress, pharmacological, or heat stress preconditioning, but could be due to the fact that the authors missed the window of expression by measuring mRNA immediately following exercise.

Nitric Oxide as a Mediator of Cardioprotection. The preceding paragraph outlined the role of NO as a trigger of cardioprotection, but NO also plays a role in mediating protection against I/R injury. NO production is elevated in the ischemic and post-ischemic myocardium (90, 164), and the overwhelming majority of studies to evaluate this phenomenon report that it has a protective effect upon the myocardium (17). Exogenous NO administration during perfusion has a protective effect (11, 12, 66, 75, 125), and the protective effects of pharmacological (12, 62), ischemic (20, 131), or heat-stress (1, 74) preconditioning can be blocked by NOS inhibitors. The precise mechanism by which NO protects is not known but possibilities include: inhibition of

calcium overload, activation of mitochondrial K_{ATP} channels, or antioxidant actions (see (17) for review). Recent evidence suggests that NO may exert protective effects through the activation of guanylate cyclase to produce cGMP (84). Only one study to date has examined the role of NOS in acute exercise-induced cardioprotection (4). The authors report that the iNOS-specific inhibitor aminoguanidine could block the protective effects of 21 minutes of treadmill exercise against 25 minutes of in-vivo coronary occlusion; however, the intensity of the exercise was very low, the duration of exercise was very short, and the measure of recovery used (% survival) was highly variable. Perfusion with aminoguanidine not only decreased survival in the exercised animals but also decreased the percent survivability to 0% for the sedentary animals. Therefore, it is not clear if aminoguanidine was blocking a protective effect or causing myocardial dysfunction independent of its inhibitory effects, and any increases in NOS could be due to the highly variable method of measuring NOS enzymatic activity rather than content. The results of the present study should help to clarify the role of NOS in acute exercise-induced cardioprotection.

Justification for Experimental Parameters. During the course of the present study two non-physiological inhibitors are used to elucidate the triggers and mediators of acute exercise-induced preconditioning and the concentrations of inhibitors used are based upon the results of previous investigations. MPG is selected based upon its ability to scavenge •OH *in vivo* (19) and *in vitro* (148), and its ability to readily cross cell membranes (44). MPG is injected into animals at a concentration of 100 mg/kg prior to treadmill running, with the intention of blocking the triggering of preconditioning

provided by ROS, which is consistent with studies observing an abrogating effect of MPG upon either heat-stress (2, 174), ischemic (156, 160), or exercise (173) preconditioning. Following an IP injection of MPG, the concentration reaches a maximum concentration 15 minutes following injection, and will decrease to 50% by one hour (44), which allows for the concentration to remain at an appropriate level during the exercise bout to retain its scavenging capacity.

NOS is inhibited through the administration of L-NAME both during exercise and during isolated perfusion. L-NAME was selected over other NOS inhibitors (129) based on the fact that it is a competitive inhibitor of all isoforms of NOS and that it has a well documented role in the inhibition of preconditioning stimuli (1, 3, 62, 68, 168). L-NAME is injected into animals at a concentration of 50 mg/kg, 15 minutes prior to initiation of the acute exercise bout, for determination of the role of NOS in the cardioprotective effect. This concentration blocks heat-stress induced preconditioning (3), while lower (68, 87) and higher (56, 97, 168, 176) concentrations successfully inhibit NOS in other exercise studies. For determination of the role of NO in mediating preconditioning, L-NAME is included in the perfusate at a concentration of 100 μM, which blocks the preconditioning effects of either pharmacological (12, 62), ischemic (131), or heat-stress preconditioning (74). It has been reported that 100 μM is the minimum concentration required for 100% inhibition of NOS (126), and further support for the use of 100 μM L-NAME is found in studies reporting that 100 μM L-NAME does not alter the response to I/R injury in the absence of a preconditioning effect (162),

and that extremely low concentrations (2 μM) may actually improve recovery from I/R injury (83).

Although we have found previously that a single day of exercise is sufficient to provide a cardioprotective effect (145, 158), 2 days of exercise was selected for this study. This selection is based on the observations of some investigators that the protective effect of acute exercise peaks 48 hours after a single exercise bout or following other preconditioning stimuli (76, 173). Therefore, two bouts of exercise assure that all of the protective agents are in place. The rats are run for an hour at a speed of 20 m/min up a 6-degree grade, on both days, which represents approximately 75% of their maximal aerobic capacity (VO_{2max}) (25). 2 days of exercise will still allow evaluation of the acute exercise phenomenon; however, it will fall into a period of late-phase recovery overlapping other various exercise interventions found in the literature. The series of experiments described herein attempt to address the question of what are the triggers and mediators of acute-exercise induced cardioprotection, as it has already been unequivocally established that acute exercise can precondition the myocardium against many different types of damage.

Subject Description

The subjects in this experiment are Male Fischer 344 (F-344) rats obtained from the National Institute on Aging (NIA) and housed on the campus of The University of Texas at Austin. The rats were 4-6 months old and weighed between 300-350 g (Table 1), and were divided into nine different treatment groups: sedentary (SED); two days of treadmill exercise at 20 m/min, 6-degree grade, for 60 minutes (RUN); sedentary / injected with 100 mg/kg MPG (a potent ROS scavenger) (SED/MPG); exercise / injected with MPG (RUN/MPG); sedentary / perfused with 100 μM L-NAME (a competitive inhibitor of NOS) (SED/L-N); exercise / perfused with L-NAME (RUN/L-N); exercise in a 4°C environment (to prevent HSP70 increase) / perfused with L-NAME (CRUN/L-N); sedentary / injected with 50 mg/kg L-NAME (SED/-NOS); exercise / injected with L-NAME (RUN/-NOS).

Questions and Hypothesis

The purpose of the experiments outlined herein is to answer four primary questions. In answering these questions, observations of a number of expected changes in cardiac function and protein expression will be made, which are as follows:

Question 1) Will 2 days of treadmill exercise in rats induce a cardioprotective effect mediated by an uncoupling of mitochondrial respiration? If so, the following changes may occur:

1) A decrease in the cardiac efficiency (COxSP/O_2 consumption), prior to and 30 minutes following ischemia in RUN vs. SED.

2) An increase in the concentration of uncoupling protein 2 (UCP2) in the myocardium of RUN vs. SED.

Question 2) Will the production of ROS in the myocardium during exercise trigger the cardioprotective effect of 2 days of treadmill exercise in rats? If so, the following changes may occur:

1) Recovery of cardiac function (COxSP), 30 minutes following I/R will be greater in RUN vs. SED. This improvement in recovery will be blocked by the administration of MPG prior to exercise in RUN/MPG.

2) The expression of various cardioprotective proteins will increase in RUN vs. SED including: HSP70, CAT, MnSOD, iNOS, nNOS, and eNOS. These increases in protein expression will be blocked by the administration of MPG prior to exercise in RUN/MPG.

Question 3) Will the production of NO during exercise trigger the cardioprotective effect of 2 days of treadmill exercise in rats? If so, the following changes may occur:

1) Recovery of cardiac function (COxSP), 30 minutes following I/R will be greater in RUN vs. SED. This improvement in recovery will be blocked by the administration of L-NAME to the animals prior to exercise in RUN/-NOS.

2) The expression of various cardioprotective proteins will increase in RUN vs. SED including: HSP70, CAT, MnSOD, iNOS, nNOS, and eNOS. These increases in protein expression will be blocked by the administration of L-NAME to the animals prior to exercise in RUN/-NOS.

Question 4) Will the production of NO by NOS during I/R mediate the cardioprotective effect of 2 days of treadmill exercise in rats? If so, the following changes may occur:

1) Recovery of cardiac function (COxSP), 30 minutes following I/R will be greater in RUN vs. SED. This improvement in recovery will be blocked by the administration of L-NAME during perfusion in RUN/L-N and CRUN/L-N.

Methods

Animals and training protocols. Male 4-6-month-old Fischer 344 (F-344) rats were obtained from the National Institute on Aging (NIA) and kept at the University of Texas Animal Resource Center. The animals were maintained on a 12 h:12 h light:dark cycle and fed *ad libitum* with Harlan Teklad 7013, NIH-31 diet. Prior to experimentation, rats were randomly assigned to nine different treatment groups: sedentary (SED); two days of treadmill exercise (RUN); sedentary / injected with 100 mg/kg MPG (a potent ROS scavenger) (SED/MPG); exercise / injected with MPG (RUN/MPG); sedentary / perfused with 100 μM L-NAME (a competitive inhibitor of NOS) (SED/L-N); exercise / perfused with L-NAME (RUN/L-N); exercise in a 4°C environment (to prevent HSP70 increase) / perfused with L-NAME (CRUN/L-N); sedentary / injected with 50 mg/kg L-NAME (SED/-NOS); exercise / injected with L-NAME (RUN/-NOS). RUN rats were run on a motorized treadmill (Collins, Braintree, MA), at room temperature, for two consecutive days, 60 minutes/day at a speed of 20 meters/minute up a 6-degree grade. CRUN animals were run on the same motorized treadmill with wet fur in an environmental chamber maintained at 4°C, at the same intensity and duration as animals run at room temperature. Rectal temperature was monitored during the treadmill exercise in both the RUN and CRUN groups with a Type J Thermocoupler (no. 600-1000, Barnant, Barrington, IL). Groups receiving injections of either MPG or L-NAME received a single intraperitoneal injection of 100 mg/kg body weight and 50 mg/kg body weight, respectively, 15 minutes prior to each

bout of exercise or, in the absence of exercise, 48 and 24 hours prior to evaluation of cardiac function. All of the animals were sacrificed 24 hours after their last exercise bout or treatment. This investigation, approved by the University's Animal Care and Use Committee (detailed description of animal use in Appendix A), conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Isolated heart perfusions. Cardiac function was evaluated using an isolated, working heart preparation (119) perfused at 37°C with a modified Krebs-Henseleit buffer (preparation detailed in Appendix B) containing (in mM): 10 glucose, 1.75 $CaCl_2$, 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 24.7 $NaHCO_3$, 0.5 EDTA, 12 mU/mL insulin, and gassed with 95% O_2-5% CO_2. Animals were anesthetized with a single intraperitoneal injection of 40 mg/kg body weight of sodium pentobarbital, and 100 IU of heparin injected into the inferior vena cava. Once hearts were rapidly excised, they were weighed in ice cold saline and mounted on the perfusion apparatus, diagrammed in Figure 1. Hearts were initially perfused for 10 minutes in a non-recirculating retrograde, or Langendorff, mode at a perfusion pressure of 80 mmHg. After 15 minutes of stabilization, hearts were switched to working heart perfusion mode. Working heart function was evaluated at an atrial filling pressure of 12.5 mmHg and an afterload set by an 80 cm high aortic column (ID 3.18 mm). Groups perfused with L-NAME had solid L-NAME (no. N-5751, Sigma Aldrich, St Louis, MO) added to the perfusate prior to perfusion resulting in a final concentration of 100 μM. Coronary flow (CF) and aortic flow (AF) were determined by timed collection of the effluent dripping off the heart and

aortic column overflow, respectively, with cardiac output (CO) determined as the sum of CF and AF, and cardiac external work (COxSP) defined as the product of CO and peak systolic pressure (SP). All values were normalized for heart wet weight. Aortic pressure and heart rate (HR) were monitored through the use of a Gould DTX pressure transducer (Gould cardiovascular products, Oxnard, CA) attached to an aortic sidearm. The data from the pressure transducer was acquired on a Dell Dimension Desktop computer (Dell, Round Rock, TX), and analyzed using Bio Bench data acquisition software (National Instruments, Austin, TX). The perfusion/data acquisition system was calibrated daily against a known column of perfusate at 0 mmHg and 80 mmHg. Hearts were allowed to initially develop their own intrinsic HR; however, if this rate was lower than 270 beats/minute, they were electronically paced at 295 beats/minute with an electronic stimulator (no. 611, Phipps & Bird Inc., Richmond, VA). During the entire perfusion, the coronary effluent was pumped with a peristaltic pump (no. 7553-20, Cole Parmer Instrument Company, Chicago, IL) at a constant rate from a enclosed water jacketed chamber past a Clark-type oxygen electrode and O_2 amplifier (University of Pennsylvania Biomedical Instrument Shops, Philadelphia, PA) for the measurement of oxygen consumption as described by Starnes et al. (146). The rate of oxygen consumption (μmol/min/g heart wet weight) was calculated as: (arterial [O_2] – coronary effluent [O_2]) x rate of coronary flow. Samples of coronary effluent were collected every 5 minutes for analysis of lactate dehydrogenase (LDH) content as an indicator of sarcolemma damage. After switching from Langendorff to working mode, hearts were perfused for 15 minutes prior to cross-clamping both the atrial inflow line and aortic

outflow line to induce global ischemia. During ischemia the hearts were enclosed in a sealed water-jacketed chamber maintained at 37°C by a Poly Temp heat pump (no. 80, Polyscience, Niles, IL), which pumps water through water-jacketed tanks and lines. After 22.5 minutes, the aortic inflow was opened and the heart was allowed to recover in Langendorff mode for 10 minutes, followed by 20 minutes in the working mode. At the end of the perfusion period, the beating hearts were freeze-clamped and stored at -80°C until further analysis. Recovery of cardiac function (COxSP) is expressed as the percentage of pre-ischemic function (COxSP) recovered 30 minutes following the reintroduction of coronary flow post-ischemia. Preliminary studies were carried out to determine whether the decline in function following I/R injury was due to the period of ischemia and not due to a general decline in function with advancing perfusion time. The results of these preliminary studies demonstrate that cardiac function, in the absence of ischemia and reperfusion, does not decline during 60 minutes of perfusion (Figure 3); therefore, all experimental interventions employed were assessed with confidence that any declines in function were due to the ischemic intervention.

Lactate dehydrogenase Assay. Coronary effluent samples collected every five minutes during the perfusion were analyzed for LDH release using a standard kinetics assay (13) of the rate of decline in NADH absorbance at 340 nm as lactate is generated from pyruvate under non-limiting substrate conditions, detailed in Appendix C. Elevated LDH release indicates that the sarcolemma has become damaged and is one of the most widely used markers of tissue injury. Although necrosis will certainly result in cytosolic enzyme leakage (138, 178), initial occurrence may precede evidence of

necrosis (147). Preliminary studies (Figure 4) demonstrated that LDH release does not increase following 60 minutes of non-ischemic perfusion.

Tissue Homogenization. For all assays of enzyme activity and protein content, a piece of left ventricle (130-160 mg) was homogenized (1:20 wt/vol) in phosphate buffer (50 mM K_2HPO_4, 0.1 mM EDTA, 0.1% Triton X-100, pH 7.4) using a Teflon-glass Potter-Elvehjem homogenizer immersed in an ice bath, detailed in Appendix D. The protein concentration of the homogenate was determined by the method of Lowry et al (99), detailed in Appendix E.

Affinity Chromatography. The concentrations of iNOS and nNOS in the whole tissue homogenate were too low to detect when directly loaded onto a gel; therefore, 1 mL of 1:20 tissue homogenate was purified through the use of 2'5' ADP Sepharose as described by Harris et al. (60), detailed in Appendix F.

SDS-PAGE and Western Blotting. Samples were loaded as described in Appendix G, after having been appropriately diluted with Laemmli (88) sample buffer. Subsequently, the samples were subjected to SDS-PAGE, detailed in Appendix G, and blotted, detailed in Appendix H and I, with the optimized concentrations of antibodies for each protein measured, detailed in Appendix J. The content of the various proteins measured in the myocardium is reported as a percentage of standard heart homogenate loaded on each gel and is adjusted for the protein concentration of the sample homogenate. All experimental blots are within a linear region for their respective concentrations.

Catalase and Superoxide Dismutase activity determinations. Homogenates were centrifuged at 1,500 X g for 10 minutes at a temperature of 4°C. The supernatant was kept on ice and analyzed for catalase activity polarographically within 2 hours using a Clark-type oxygen electrode according to Del Rio et al. (41), detailed in Appendix K. MnSOD activity was measured spectrophotometrically according to McCord and Fridovich (105), detailed in Appendix L. Enzyme assays were run at 25°C.

Statistical Analysis. Descriptive data (means ± SE) are calculated for each dependent variable. For comparisons a factorial analysis of variance (ANOVA) was used to test for overall significance, with a Tukey's HSD test used for post-hoc analysis. In all tests, a probability level of <0.05 is used as the decision rule for significance testing. The raw data for all variables measured is listed in Appendix M.

Limitations

In this study, an animal model was used to represent the metabolic activity of humans which limits the applicability of the findings. Factors, such as catecholamine release and other metabolic responses to physical stress, may have resulted in changes that are not be accounted for in the current study. Cardiac function is measured as the product of cardiac output and the peak systolic pressure. These variables may be susceptible to measurement error and misrepresentation; however, this product is widely accepted as a true measure of hydraulic work and is commonly used in our laboratory. During the short period (<2 minutes) after the heart is removed, until it is supplied with perfusate, the heart was ischemic and short periods of ischemia may provide a cardioprotective effect by preconditioning the heart against a subsequent ischemic stress (see (177) for review). However, the heart was kept in ice-cold saline during this period of time, which should have minimized any ischemic damage or triggers of preconditioning and the hearts of all experimental groups experienced the same treatment. Core temperature was measured by insertion of a rectal thermometer. This measure of core temperature is not as precise as direct measurement of cardiac temperature, but is used as a close approximation. A number of non-physiological inhibitors were injected into the animals and included in the perfusate of some of the various treatment groups. These inhibitors may have unintended metabolic consequences in the myocardium that cannot be accounted for. Additionally, the

absorption of these inhibitors into the myocardium may be limited or incomplete, resulting in reduced or partial inhibition of the intended targets.

Delimitations

The study was executed with male Fisher-344 rats, 4-6 months old and weighing 300-350 g. This limits applicability to humans, but is used as a generalization of metabolic activity and cardiac function in all mammals.

Results

GENERAL OBSERVATIONS

Animal Characteristics. The body weights, heart weights, and ratio of the two are reported in Table 1. There were no significant differences among any of the groups with respect to body weight or heart weight ($P>0.05$). The only variation found within these variables was in CRUN/L-N, which had a slightly lower body weight/heart weight ratio ($P<0.05$) due to an insignificantly ($P>0.05$) lower body weight and higher heart weight. Although the exercise in the present study is acute, a similar deviation has been observed in animals that were chronically run in the cold (61). Although heart weight was not increased in the present study to significant levels ($P>0.05$), and to a lesser degree than in the chronic study, similar mechanisms acting over a shorter duration may be involved. The cause of the increase is unclear, but could be due to an increase in the total peripheral resistance resulting from increased vasoconstriction during exercise in the cold as was described by Harris and Starnes (61). This increase was not found in my previous study (158), potentially because of the fewer bouts of exercise (1 vs. 2), or to differences in core temperature discussed in the following section.

Core Temperature. The core temperatures of the animals while at rest and during exercise (30 and 60 minutes into the bout of exercise) are reported in Table 2. The temperature of the RUN animals was significantly ($P<0.05$) higher than SED rest at all time points and was significantly ($P<0.05$) lower at all time points in CRUN/L-N. The lower temperature in CRUN/L-N was not expected to be lower than SED rest and

although this may have affected the body weight/heart weight ratio, it does not appear to have affected the functional results, as discussed in following sections.

QUESTION 1 – UNCOUPLING AS A MEDIATOR

Cardiac Function. No significant differences in cardiac efficiency (COxSP/oxygen consumption) were observed in any group prior to ischemia ($P>0.05$); however, efficiency was higher in RUN vs. SED at all time points following ischemia (Figure 5).

UCP2 Expression. The concentration of UCP2 in the whole heart homogenate was too low to detect by western blotting (data not shown).

QUESTION 2 – ROS AS A TRIGGER

Cardiac Function. Hemodynamic parameters prior to and 30 minutes following ischemia are displayed in Table 3. Prior to ischemia, cardiac function (COxSP) (Figure 6) was similar in all groups except SED/MPG, which was 15.0% higher than RUN/MPG; therefore, COxSP following ischemia was evaluated as a percentage of the pre-ischemic function (Figure 7). Following ischemia, RUN recovered a greater percentage of pre-ischemic function than SED ($P<0.05$), MPG alone did not affect recovery (SED vs. SED/MPG, $P>0.05$), and MPG did not block the preconditioning effects of exercise (RUN vs. RUN/MPG, $P>0.05$). No differences in cardiac efficiency were observed prior to ischemia ($P>0.05$) and although efficiency tended to be higher in RUN and RUN/MPG following ischemia, only RUN reached the ($P<0.05$) level of significance at 20 minutes post-ischemia (data not shown). Release of LDH (Figure 8)

was not different in any group prior to ischemia ($P>0.05$) and increased in all groups 5, 10, and 15 minutes following ischemia ($P<0.05$). The magnitude of the increase was greatest at 10 minutes post-ischemia, and training status was more important in reducing sarcolemma damage as MPG injection did not significantly ($P>0.05$) affect LDH release (SED vs. SED/MPG, RUN vs. RUN/MPG), while at 10 and 15 minutes post-ischemia LDH release was greater in SED vs. RUN ($P<0.05$).

Tissue Analysis. Expression of HSP70 in the left ventricle (Figure 9) increased in RUN vs. SED ($P<0.05$), was not affected by MPG alone (SED vs. SED/MPG, $P>0.05$), and was not blocked by administration of MPG (RUN vs. RUN/MPG, $P>0.05$). No significant changes in the expression of iNOS (Figure 10) or eNOS (Figure 11) were detected in any group ($P>0.05$), and nNOS expression could not be detected any experimental sample. This may be due to species-specific differences in expression, as nNOS could be detected in the myocardium of Sprague Dawley rats, but not in samples from the F-344 rats used in the present study (Figure 12). The activity of CAT (Figure 13) increased in groups SED/MPG and RUN/MPG vs. SED and RUN ($P<0.05$), which suggests that MPG alone induced an increase in CAT; however, this increase in CAT did not affect tolerance to I/R injury (Figure 7). No changes in MnSOD activity (Figure 14) were observed in any group ($P>0.05$).

QUESTION 3 – NO AS A TRIGGER

This question was not able to be addressed, as the animals were not able to perform the exercise after receiving injections of L-NAME. The reasons for this observed phenomenon are discussed in detail in the following sections.

QUESTION 4 – NO AS A MEDIATOR

Cardiac Function. Hemodynamic parameters prior to and 30 minutes following ischemia are displayed in Table 3. Of these parameters the most important observation is that perfusion with L-NAME decreased CF (SED pre vs. SED/ L-N pre, RUN pre vs. RUN/L-N pre, $P<0.05$), suggesting that NOS was effectively inhibited. Prior to ischemia, cardiac function (COxSP) (Figure 15) was not different between any group ($P>0.05$). Thirty minutes following ischemia, RUN recovered a greater percentage of pre-ischemic function than SED ($P<0.05$), L-NAME alone did not affect recovery (SED vs. SED/L-N, $P>0.05$), and L-NAME did not block the preconditioning effects of exercise in the ambient temperature (RUN vs. RUN/L-N, $P>0.05$) or in the cold (RUN vs. CRUN/L-N, $P>0.05$). No differences in cardiac efficiency (Figure 16) were observed prior to ischemia ($P>0.05$). Following ischemia, at all time points, all exercise groups (RUN, RUN/L-N, and CRUN/L-N) displayed greater efficiency ($P<0.05$) than both sedentary groups (SED and SED/L-N), which was not affected by L-NAME under any circumstances. Release of LDH (Figure 17) was not different in any group prior to ischemia ($P>0.05$) and increased in all groups, except CRUN/L-N, 5 and 10 minutes following ischemia ($P<0.05$). The magnitude of the increase was greatest at 10 minutes post-ischemia, while exercise in ambient or cold temperature reduced release in all groups and was not increased by L-NAME (RUN, RUN/L-N, and CRUN/L-N vs. SED, $P<0.05$). Perfusion with L-NAME alone appears to protect against sarcolemma damage as LDH release was reduced in SED/L-N vs. SED ($P<0.05$), but this protective effect was not manifested into greater functional recovery (Figure 15).

Tissue Analysis. Expression of HSP70 in the left ventricle (Figure 18) increased in RUN vs. SED ($P<0.05$), and was not affected by administration of L-NAME (SED vs. SED/L-N; RUN vs. RUN/L-N and CRUN/L-N, $P>0.05$). No significant changes in the expression of iNOS (Figure 19) were observed ($P>0.05$) and eNOS (Figure 20) was elevated in CRUN/L-N vs. all other groups ($P<0.05$), which may explain the increased CF observed in my previous study (158), but does not appear to be the primary mediator of the cardioprotection observed as L-NAME did not block the cardioprotective effect of acute exercise in the cold (Figure 15). No changes in CAT (Figure 21) were observed in any groups ($P>0.05$); however, MnSOD (Figure 22) decreased in CRUN/L-N vs. SED and RUN ($P<0.05$).

Discussion

GENERAL OBSERVATIONS

The preliminary studies that led to the initiation of these experiments (158) demonstrated that acute exercise-induced cardioprotection could be acquired without increasing HSP70, which was previously thought to mediate the improved tolerance to ischemia reperfusion injury (98). The present study represents the investigation of a number of other potential triggers and mediators of acute exercise-induced cardioprotection, including ROS and NO, that have been implicated in heat-stress and ischemic preconditioning. As will be discussed below, there appear to be numerous similarities in how the various forms of preconditioning manifest into cardioprotective phenotypes; however, the results of our experiments suggest that important differences exist between acute exercise preconditioning and those from heat-stress or ischemic preconditioning.

QUESTION 1 – UNCOUPLING AS A MEDIATOR

Induction of an uncoupling effect following acute exercise appears to be counterintuitive, as one would expect that exercise would induce changes increasing the efficiency of myocardial hydraulic work; however, non-physiological uncouplers clearly do protect against I/R injury (111). If acute exercise were to induce these changes, cardiac efficiency should be expected to decrease both before and after, or potentially only after ischemia if the activity of the uncoupling protein were to be

activated by increased production of ROS and/or other stresses. Prior to the present investigation none of these variables had been evaluated following acute exercise. The following results suggest that uncoupling does not mediate acute exercise-induced preconditioning.

In the present study, cardiac efficiency prior to ischemia was similar in all groups; however, throughout the post-ischemic recovery period hearts of animals run in either the ambient temperature or in the cold functioned much more efficiently than hearts of sedentary animals (Figures 5 and 16). If uncoupling of oxygen consumption from ATP production occurred in any animals, it was the sedentary groups whose post-ischemic recovery of mechanical function was considerably less than that of the runners. In the SED group, post-ischemic function decreased 49.7% compared to the pre-ischemic value, but oxygen consumption only declined 15.6%. Therefore, the greater proportional decrease in function as compared to oxygen consumption in SED resulted in the lower ratio of cardiac efficiency.

Although the effects of L-NAME will be discussed in greater detail in following sections, it should be noted that L-NAME in the perfusate did not affect myocardial oxygen consumption or efficiency (Figure 16) in spite of a significant decrease in CF in SED/L-N vs. SED (Table 3). Normally CF will increase/decrease in direct proportion to the oxygen demands of the myocardium. In the isolated, buffer perfused, working rat heart, the oxygen concentration in the coronary flow effluent is relatively high (approximately 325 μM), providing for considerable O_2 reserve to allow for increased O_2 extraction when CF rate is reduced by NOS inhibition. It was observed that when L-

NAME was present in the perfusate, O_2 extraction increased in proportion to the decrease in CF and the O_2 concentration in the coronary effluent dropped to approximately 175 μM. It is unlikely that this much flexibility in CF and O_2 extraction exists *in-vivo* where the baseline CF rate is much lower and O_2 in the blood is bound to hemoglobin. The kinetics of oxygen extraction in the hemoglobin-free perfusion buffer is not limited by the oxygen saturation characteristics and carrying capacity of hemoglobin.

The western blotting experiments, initiated in order to detect changes in UCP2 expression following the various exercise protocols and interventions, did not yield any reliable results due to the fact that the concentration of the protein was too low to detect in whole heart homogenate samples. Knowing that the site of action for UCP2 is the mitochondria, it is possible that the protein could have been detected in samples of isolated mitochondria; however, the hearts had already been frozen, thereby prohibiting the isolation of the mitochondria. Recently, another group of investigators have reported that they were able to detect UCP2 protein in mitochondria isolated from cardiac tissue (106). The authors provide substantial evidence that ischemic preconditioning, which provides a preconditioning effect in the myocardium against I/R injury, was capable of increasing the expression of UCP2 two-fold and that this increase was primarily responsible for mediating their observed cardioprotection. These data would appear to conflict with the findings of the present study and this is the first of many differences to be contrasted in this discussion regarding acute exercise-induced vs. other preconditioning stimuli. However, due to the fact that cardiac efficiency was not

decreased, but rather increased, in all exercising groups, it is not likely that uncoupling serves to mediate acute exercise-induced preconditioning.

QUESTION 2 – ROS AS A TRIGGER

The results presented herein suggest that quenching ROS during exercise does not block the trigger that initiates the adaptations leading to acute exercise-induced cardioprotection. This is represented by the inability of MPG administration during two days of exercise to decrease the amount of post-ischemic functional recovery compared to exercise without MPG administration (RUN/MPG vs. RUN) (Figure 7). The administration of exogenous MPG as a radical scavenger has been used in experimental studies for many decades now (19, 45, 112, 113, 133, 141), and the dosage of 100 mg/kg selected for the present study is known to block the triggering stimuli of both heat-stress (2, 174) and ischemic (156, 160) preconditioning. One other group of investigators (173) reported that 100 mg/kg MPG administration during a single 20-minute exercise bout prevents acute exercise-induced cardioprotection, 48 hours following cessation of exercise, against infarct development following coronary artery occlusion *in vivo*. They propose that the acute exercise-induced cardioprotection is due to increased MnSOD (67% increase 48 hours after exercise) and they report that MPG prevented up-regulation of this antioxidant enzyme. It should be noted that no other studies have reported such a dramatic change in MnSOD even when the exercise bout is longer and more intense. The differences between the results of Yamashita et al. (173) and those presented herein could be due to either the strain of animal (Wistar), the indices of myocardial dysfunction used (infarct size), or to the short duration of exercise

(20 minutes). In any case, this same group of investigators regularly report results that differ than those of many other investigators; therefore, the work discussed herein should help to clarify the effect of MPG upon acute exercise-induced cardioprotection. Prior work with this dosage of MPG has established that it may result in mild liver damage (133). For this reason, and due to the fact that the 100 mg/kg dosage has been effective against several forms of preconditioning, it would not be reasonable to assume that the dosage could have been further increased in order to augment the scavenging capacity.

Included among the many functional parameters listed in Table 3 is that 10-12 rats were perfused in answering the question regarding ROS as a trigger (SED/MPG and RUN/MPG), while only 6-7 rats were required to reach significance in all other experimental groups. The reason for the additional MPG animals was that a high variability in the absolute values of post-ischemic function was observed in these groups. We subsequently realized that the variability during post-ischemia was due to slight differences in the pre-ischemic COxSP in these two groups. When the COxSP values for SED/MPG and RUN/MPG were scrutinized, the observation was made that the variations in post ischemic values paralleled those of the pre-ischemic on a reasonably individual basis, thus it appeared that there was a degree of covariance between the pre- and post-ischemic variables. When the post-ischemic recovery was expressed as a percentage of pre-ischemic function in each heart, the variability decreased and it became apparent that MPG administration did not prevent improved acute exercise-induced cardioprotection. The results for LDH release (Figure 8), which

show no main effect of MPG, only serve to validate the present interpretation of the functional results. Therefore, the sum of the observations, including the additional data points for the functional data and the LDH results, increases confidence in the conclusion that MPG did not inhibit the triggering response of acute exercise and that MPG did not induce a protective effect in sedentary animals.

The reason for the MPG-related alterations in pre-ischemic COxSP is not clear; however, initial measurements of enzymatic activity suggested that injection of MPG was causing some other unusual adaptations, including an increase in CAT activity independent of training status (Figure 13). The rationalization initially proposed as an explanation for the increased catalase activity was that there was residual MPG in the myocardial tissue and it was somehow affecting the measurement of CAT. This explanation was dismissed by adding MPG directly to the reaction vessel during analysis to observe its affect on the measurement of CAT in an untreated sample. No changes in the assay rate were observed with added MPG (data not shown), which agrees with earlier reports that MPG does not affect H_2O_2 metabolism (19). The mechanism for increased CAT activity is not apparent; however, a similar response has been observed in hepatic tissue (52), but not in cardiac tissue with chronic administration/training (40). It is important to note that administration of MPG did not have the effect of inhibiting or increasing any of the other cardioprotective proteins, including the increase in HSP70 due to two days of running (Figure 9).

Although no other differences in protein expression resulted from MPG administration, an unexpected species-specific variation was inadvertently discovered.

Blotting for nNOS initially was not providing any results; however, it was noticed that a band was appearing at the appropriate molecular weight in the lane corresponding to the standard that was loaded. After checking the records regarding the standard, a realization was made that the standard was composed of a pooled homogenate from Sprague Dawley rats vs. F344 used in the present study. A qualitative comparison of nNOS expression between Sprague Dawley and F344 rats (Figure 12) shows that the expression of nNOS in sedentary animals is much lower in F344 and is not up-regulated in response to acute exercise. The levels of protein were too low to quantify and; therefore, are not statistically analyzed. nNOS does not appear to have a significant role in the myocardium of F344 rats, but the differences described herein could explain some of the differences reported in the literature regarding NOS.

QUESTION 3 – NO AS A TRIGGER

As stated in the results section, this question was not able to be addressed, as the rats were not able to complete the exercise bout. Specifically, following the initiation of exercise, the rats could not run more than ~10 minutes before requiring excessive use of a motivational stimulus. It was determined, after consulting with my supervising professor, that continuation of the exercise protocol would be inhumane. The options available for adapting our treatment protocol were discussed including: decreasing the amount of exercise time and/or the concentration of L-NAME administered. With regards to the duration of exercise, it was decided that decreasing the amount of running time was not a viable option. If a decreased tolerance to I/R following two shorter bouts of exercise were observed, it would not be clear if the observed effects were due to

inhibition of the triggering stimuli by L-NAME, or simply a lack of a stimulus of a sufficient magnitude to trigger cardioprotection.

It has been reported that L-NAME can be administered prior to treadmill exercise in rats, but the dosages used to date are well below what is required for complete inhibition of NOS. For example, Musch et al. (118) and Husain (67) used a dose of 10 mg/kg, which is 5 times lower than the dose used in the present study. This dosage would be approximately equivalent to a concentration of 37 μM (10 mg / 269.7 g/mol), assuming that it was absorbed into all tissues proportionally. However this does not happen, as muscle tissue accounts for approximately 50% of the total mass of the body, and even if the assumption was made that L-NAME were to distribute evenly throughout all muscle, the final concentration would be maximally 74 μM, ignoring degradation and absorption into other tissues. These two concentrations are 63% and 26% respectively, below what is minimally required (100 μM) for complete inhibition of NOS (126). It has been confirmed experimentally that 4 mg/kg of L-NAME results in a final skeletal muscle concentration of 38 μmol per kg of dry weight in humans, which corresponds to a measured wet tissue concentration of 10 μM (55). The authors report that this concentration will inhibit 67% of NOS activity, measured in extracted tissue samples from human skeletal muscle, when the conversion of arginine to citrulline was assayed. Assuming a linearity of increasing muscle concentration with increasing dosage, the rats from the first two studies (67, 118) would have a maximal tissue concentration of 25 μM (well below the inhibitory threshold of 100 μM) and the rats from the experiments proposed for the present study would have a maximal tissue

concentration of 125 μM (above 100 μM). Therefore, if the concentration of L-NAME were decreased in the present study, to a level permitting completion of the exercise protocol, NOS would not be completely inhibited and the triggering response, if it occurs through this pathway, would be only partially inhibited. If production of NO by NOS is responsible for triggering acute-exercise induced preconditioning, any cardioprotective effects may not be abrogated.

The underlying reason for the inability of the rats to perform extended treadmill exercise may be because of a decrease in either skeletal or myocardial muscle blood flow or a combination of both. During exercise, the oxygen demands of both cardiac and skeletal muscle increase in response to the greater rates of energy utilization. If the normal maximal capacity for oxygen to be delivered is attenuated, then the maximum rate of energy utilization, i.e. rate of exercise, will also be decreased. The dosage of L-NAME used in the present study (50 mg/kg) should not have affected resting myocardial CF or myocardial function as 30 mg/kg has been previously reported to have no effect, while 300 mg/kg can affect resting values (37). L-NAME, in dosages of 1-10 mg/kg, decreases myocardial oxygen uptake (139), while other studies have observed either no effect upon oxygen uptake in skeletal muscle with similar concentrations (55) or alternatively decreases in skeletal muscle vascular conductance (118). Other studies that provide data supporting a decreased exercise capacity due to NO alterations include: a decreased voluntary exercise response in mice with NOS knocked-out (115) and a decreased exercise capacity in humans with decreased NO production following heart transplant (136). The collective conclusion derived from

these studies is that in order for a rat to exercise at an intensity and duration demanded in the present study, adequate NO production and muscle blood flow is required.

Unfortunately, the present study does not provide an answer to the question as to whether or not NO serves as a trigger of acute-exercise induced preconditioning. In order for this question to be answered in the future, the cellular targets involved with the vasodilatory effects of NO will need to be separated from those which initiate pathways of preconditioning. In the review of literature, it is stated that nitrosylation of PKCε has been proposed as a primary target for activation of the preconditioning response (7, 153). If this signaling event could be blocked either by specifically inhibiting the actions of NO at this site of activation or by mutating the activation site on this signaling protein, thereby preventing activation, the relative contribution of NO to the triggering response of acute exercise could be elucidated. Clearly, additional work will be required to fully address this question, which lies beyond the scope of the present investigation.

QUESTION 4 – NO AS A MEDIATOR

Although NO has the ability to trigger preconditioning, it can also mediate cardioprotection (17); however, the results of this study suggest that it is not a mediator of acute exercise-induced cardioprotection against myocardial stunning. The observation that CF increased in the group of animals run in a cold environment (158) initiated the investigation of NOS as a potential mediator of acute exercise-induced cardioprotection; however, both the functional results and the protein expression patterns of the present study suggest that NOS is not a mediator of acute exercise-

induced preconditioning. L-NAME, at a concentration of 100 μM, which did not affect the pre-ischemic function of any group, was included in the perfusate both before and after ischemia (Figure 15). Following ischemia, both exercise groups receiving L-NAME recovered similarly to the exercised animals not receiving it and better than the two sedentary groups. These results suggest that NOS, irregardless of isoform, does not mediate acute exercise-induced cardioprotection. L-NAME did result in a decreased LDH release following ischemia, but this may be due to inhibition of the constitutive expression of NOS, as L-NAME decreased release in sedentary animals, SED/L-N vs. SED (Figure 17).

The absence of an abrogating effect of L-NAME is more than likely due to cardioprotective effects of other protective proteins not identified in this investigation, rather than an insufficient inhibition of NOS. The concentration of L-NAME (100 μM) used in the perfusate is the concentration required for 100% inhibition of NOS (126) and equivalent or lower concentrations have successfully inhibited the effects of ischemic (20, 131) and heat-stress (1, 74) preconditioning. Other reports show that the skeletal muscle concentration of L-NAME should exceed 100 μM (and the assumption can be made that a similar response occurs in myocardial tissue) when perfused with 100 μM (55).

The most interesting protein change to occur in this study is the increase in eNOS in CRUN/L-N (Figure 20). Although this increase in NOS did not mediate the cardioprotective effect of running in the cold, illustrated by the fact that L-NAME was not able to block the improved recovery following acute exercise, it may explain the

increased CF observed in the preliminary study that initiated this series of experiments (158). Further evidence that eNOS was responsible for the previously observed increase in CF is that L-NAME prevented a similar post-ischemic increase in CF in the present study (CRUN/L-N, Table 3).

The findings presented herein have a number of differences from those reported by Babai et al. (4) who found that the iNOS-specific inhibitor, aminoguanidine, blocked the preconditioning effects of a single, 21-minute, bout of exercise in dogs. They also found that the activity of iNOS increased 3-fold in the myocardium 24 hours following exercise. The reason for these differences from the present study could be that species-specific variations result in differential expression of iNOS, as similarly there are clearly differences in expression of nNOS in F344 rats compared to Sprague Dawley rats (Figure 12). However, a more plausible explanation can be found in how tolerance to coronary occlusion and NOS expression were evaluated. The authors report that aminoguanidine reduced survivability from coronary occlusion in exercised animals, but this same dosage of aminoguanidine also reduced survivability to 0% in sedentary animals. This suggests that aminoguanidine alone had a significant effect of lowering tolerance to coronary occlusion rather than blocking a protective effect. Conversely, in our isolated rat hearts subjected to I/R, functional recovery was not affected by L-NAME and release of LDH (an indicator of sarcolemmal damage) was decreased. The reason for these differences is not completely clear. Babai et al. detected iNOS by the highly variable assay of measuring the activity of the enzyme as arginine is converted to citrulline, rather than by western blotting or ELISA. Even in studies of heat-stress and

ischemic preconditioning, increases in iNOS are small in magnitude (17), which contrasts with the greater than three-fold increase reported by the authors; therefore, it is unclear whether or not the results can be confidently accepted.

The results of the present study represent a divergence from the reports that inhibitors of NOS are capable of blocking heat-stress, ischemic, and even acute exercise-induced myocardial preconditioning. Although eNOS was increased in the animals that ran in the cold, it was not responsible for mediating the protection provided. One of the primary differences between the results of this study and others is that iNOS did not increase under any circumstances. This could be due to the fact that the magnitude of the change was too small to detect via western blotting. However, even if there were changes that went undetected, L-NAME did not block any protective effects. Therefore, NOS is not a likely mediator of acute exercise-induced cardioprotection, and it is still not clear as to what might be the responsible for the protective effect observed herein. Adaptation to exercise is a very complex process, and depending upon the mode, duration, and intensity; different adaptations may occur. It is clear that many different proven cardioprotective proteins can be up-regulated following exercise, and the cardioprotective response may be a cumulative effect of known and unknown proteins/mechanisms, which cannot be inhibited on an individual, or even on a multiple basis as demonstrated when HSP70 was blocked by exercise in the cold and eNOS with L-NAME.

CONCLUDING REMARKS

When considered together, the results of the present study suggest that acute exercise-induced cardioprotection is not triggered by ROS production and is mediated by factors other than NOS and mitochondrial uncoupling. It is still not clear as to whether or not NO is responsible for triggering the cardioprotective effect of acute exercise; however, when the observation that NO production is necessary for performing exercise is considered together with the abundance of data supporting the role of NO to trigger a preconditioning response, it is not difficult to imagine that NO could be a trigger of acute exercise-induced preconditioning. Along with the described functional changes, increases in various cardioprotective proteins did occur; however, it is still not evident what the cumulative effects of these are and clearly there could be other cardioprotective proteins, and related changes in expression and activation, yet to be identified.

Although the various interventions employed did not abrogate either the triggers or mediators of acute exercise-induced cardioprotection, these results serve to exclude a number of proposed mechanisms. Moreover, the preconceived notion that the nature of the preconditioning repose of the myocardium to varying stimuli is uniform has been thoroughly invalidated. Although heat-stress and ischemic preconditioning may provide clues as to the mechanisms underlying the observed protection resulting from acute exercise, they do not mimic the influence of exercise exactly. Hopefully, future investigations will be able to further elucidate the mechanisms of acute exercise-

induced cardioprotection so that additional cardioprotective therapies may be developed.

XIII. Tables

TABLE 1 – ANIMAL CHARACTERISTICS

	n	Body Weight (g)	Heart Weight (mg)	Ratio (g/mg)
SED	7	336.6 ± 9.3	921 ± 25	0.366 ± 0.006
RUN	7	328.0 ± 9.6	957 ± 9	0.343 ± 0.007
SED/MPG	12	346.7 ± 4.8	918 ± 12	0.378 ± 0.005
RUN/MPG	10	341.2 ± 4.5	972 ± 16	0.352 ± 0.005
SED/L-N	6	345.0 ± 8.3	933 ± 14	0.370 ± 0.008
RUN/L-N	6	306.7 ± 3.5	905 ± 11	0.339 ± 0.003
CRUN/L-N	7	323.1 ± 10.2	990 ± 34	0.327 ± 0.004 *

Values are mean ± SE; n, number of rats. *(P<0.05) vs. SED.

TABLE 2 – CORE TEMPERATURE, PRIOR TO AND DURING EXERCISE

	Rest	30 minutes	60 minutes
SED	37.7 ± 0.1		
RUN		40.6 ± 0.1 *	41.2 ± 0.2 *
CRUN/L-N		36.2 ± 0.4 *	34.3 ± 0.6 *

Values are mean ± SE, n = 5-6. Core temperature represents rectal temperature at listed time point. *(P<0.05) vs. SED Rest.

TABLE 3 – CARDIAC FUNCTIONAL PARAMETERS

Group	CF (mL/min/g)	AF (mL/min/g)	CO (mL/min/g)	SP (mm Hg)	HR (beats/min)
			Pre-Ischemia		
SED	13.5 ± 0.5	41.3 ± 2.6	54.8 ± 2.9	114.1 ± 2.4	277 ± 9
RUN	14.2 ± 0.6	45.6 ± 1.9	59.8 ± 2.4	111.0 ± 1.4	300 ± 5 *
SED/MPG	15.4 ± 0.7	48.0 ± 1.7	63.4 ± 2.0 *	110.8 ± 1.7	291 ± 4
RUN/MPG	13.5 ± 0.5	42.7 ± 1.2	56.2 ± 1.6	108.8 ± 1.5	287 ± 5
SED/L-N	10.9 ± 0.6 *	44.1 ± 2.0	55.1 ± 2.4	108.5 ± 3.4	284 ± 6
RUN/L-N	11.9 ± 0.4	49.2 ± 1.2	61.0 ± 1.5	111.2 ± 1.1	298 ± 4
CRUN/L-N	12.4 ± 0.3	46.4 ± 1.2	58.8 ± 1.4	114.4 ± 1.8	295 ± 0
			30 Minutes Post-Ischemia		
SED	11.7 ± 0.7	19.2 ± 2.8 †	30.9 ± 2.9 †	101.3 ± 2.7 †	262 ± 13
RUN	14.2 ± 0.8 *	33.4 ± 2.7 *†	47.7 ± 3.4 *	109.1 ± 1.4	298 ± 4
SED/MPG	14.4 ± 1.0	23.7 ± 4.1 †	38.0 ± 4.4 †	99.6 ± 1.5 †	266 ± 13
RUN/MPG	13.9 ± 0.5	31.5 ± 2.5	45.4 ± 2.7	102.8 ± 1.8	285 ± 6
SED/L-N	8.7 ± 0.4 *†	19.1 ± 4.7 †	27.8 ± 5.0 †	95.3 ± 4.5	248 ± 17
RUN/L-N	11.4 ± 0.6	37.7 ± 1.8 *†	49.0 ± 2.2 *†	106.8 ± 1.5	296 ± 2
CRUN/L-N	11.7 ± 0.4	37.5 ± 1.6 *†	49.2 ± 1.4 *†	113.7 ± 1.7 *	295 ± 0

Values are mean ± SE. CF, coronary flow; AF, aortic flow; CO, cardiac output; SP systolic pressure; HR, heart rate. *($P<0.05$) vs. SED at same time point, †($P<0.05$) vs. pre-ischemic value.

Figures

FIGURE 1 - DIAGRAM OF ISOLATED-PERFUSED WORKING HEART

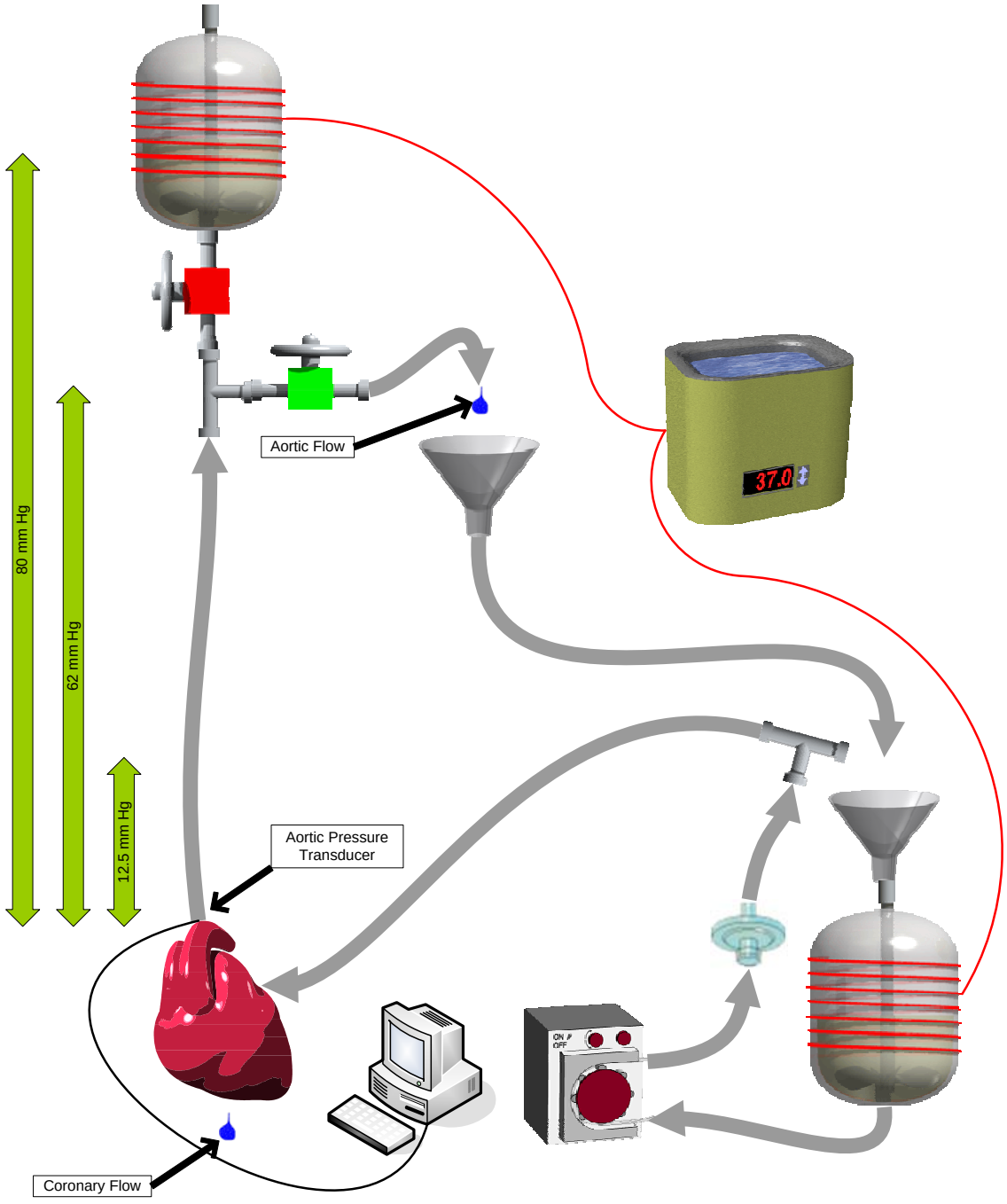


FIGURE 2 – REACTIVE OXYGEN SPECIES

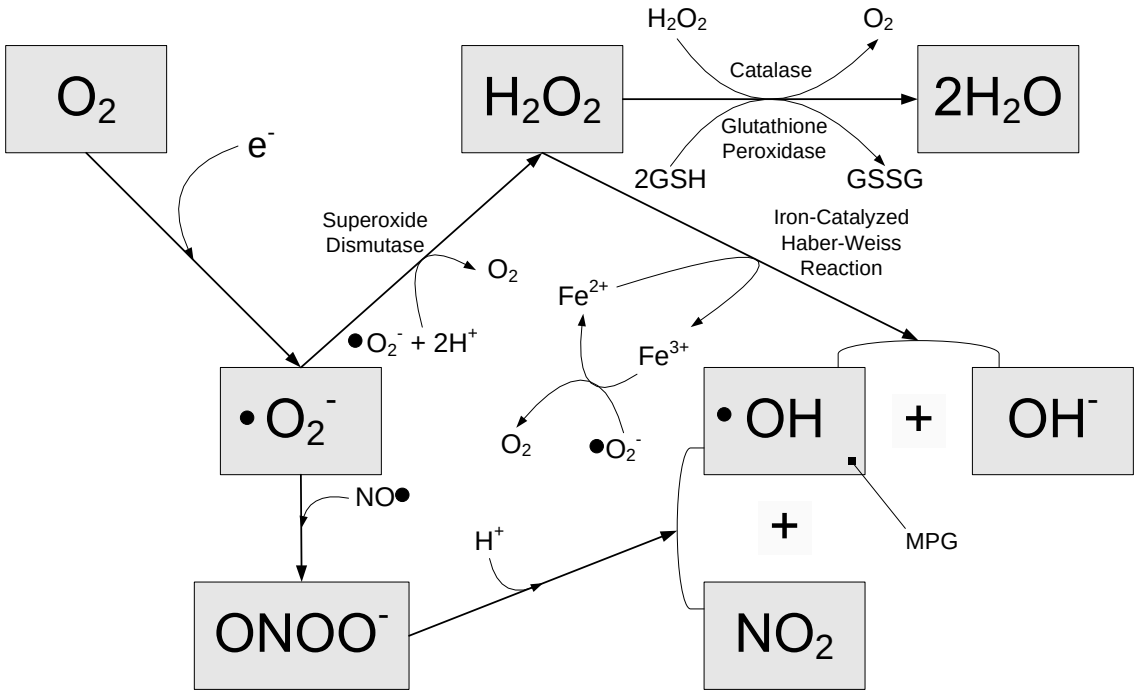
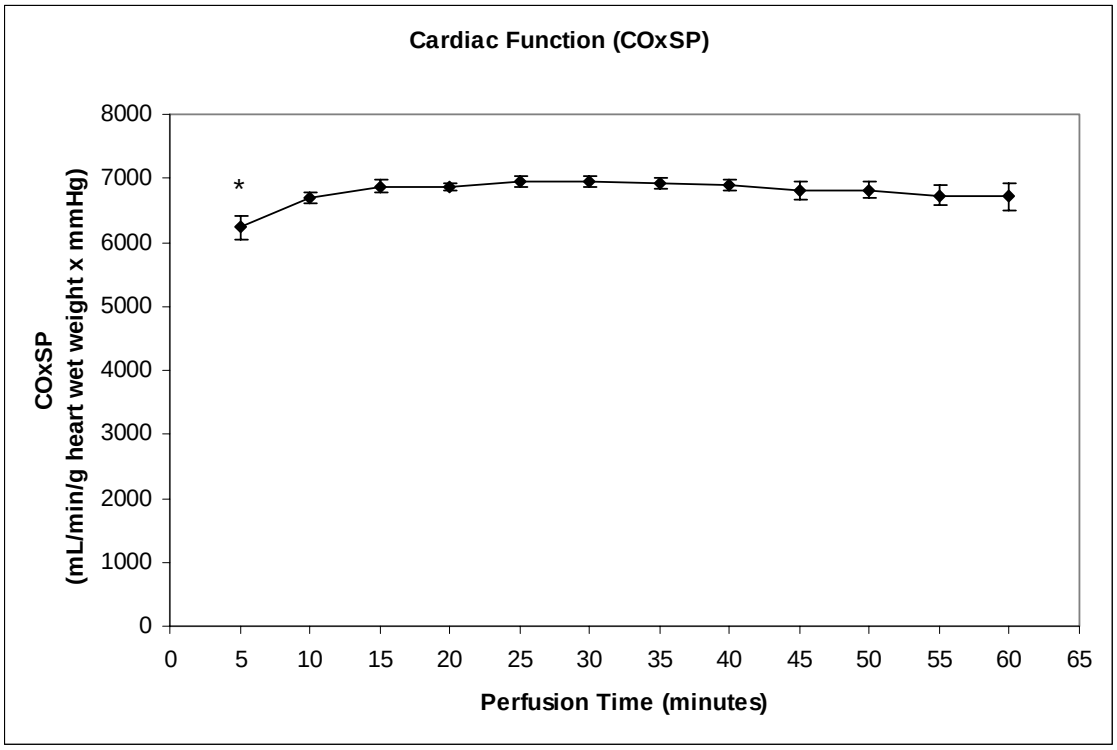
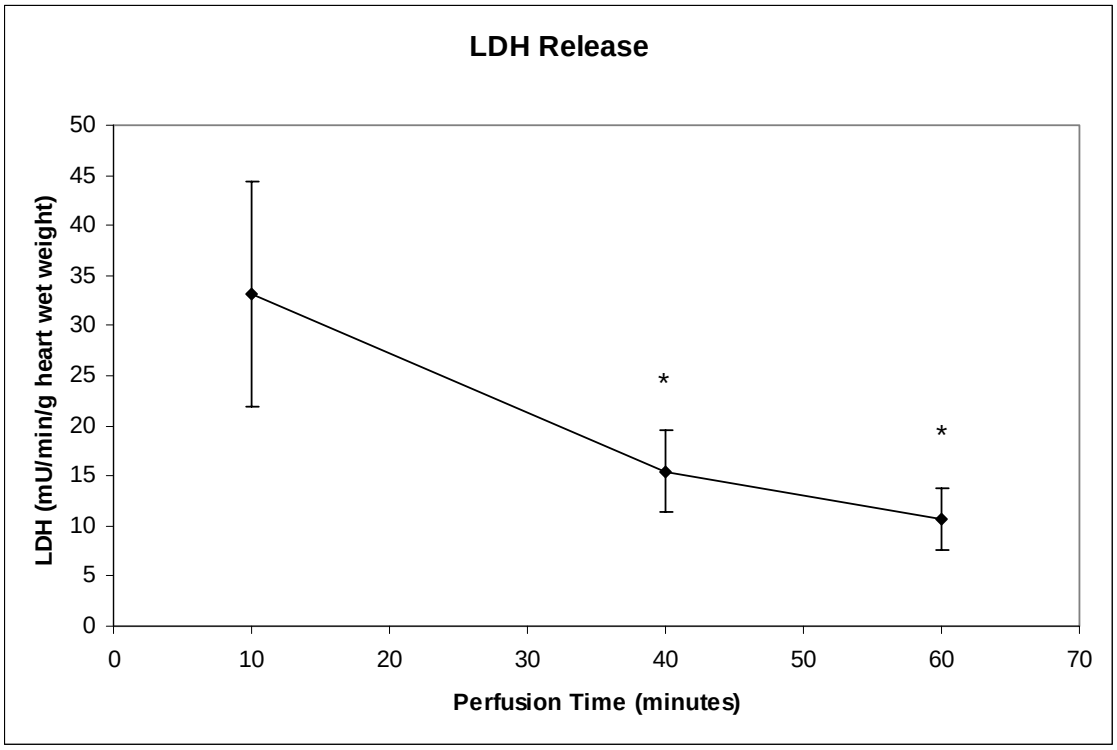


FIGURE 3 – CARDIAC FUNCTION, PRELIMINARY DATA



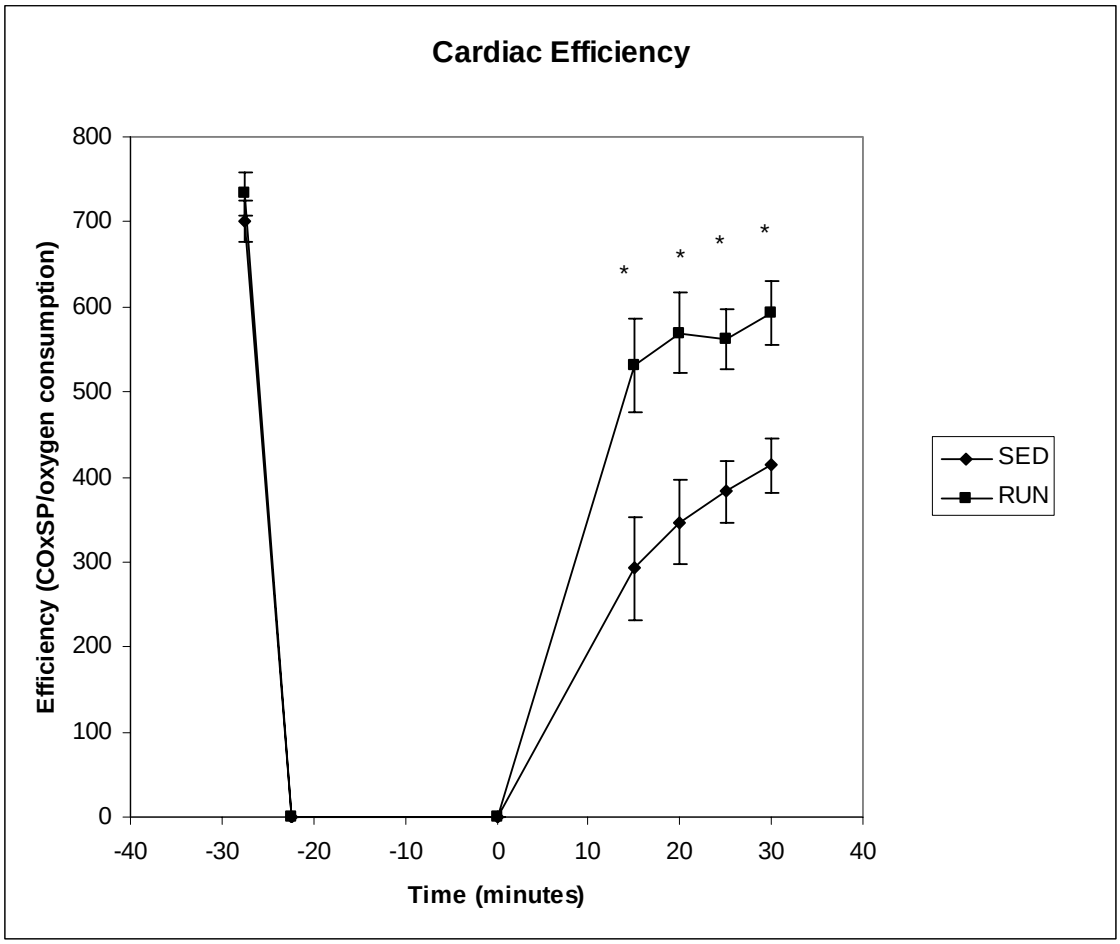
Cardiac function (COxSP) over time, in the absence of ischemia. Values are mean ± SE, n=6. *($P<0.05$) vs. 15 minutes.

FIGURE 4 – LDH RELEASE, PRELIMINARY DATA



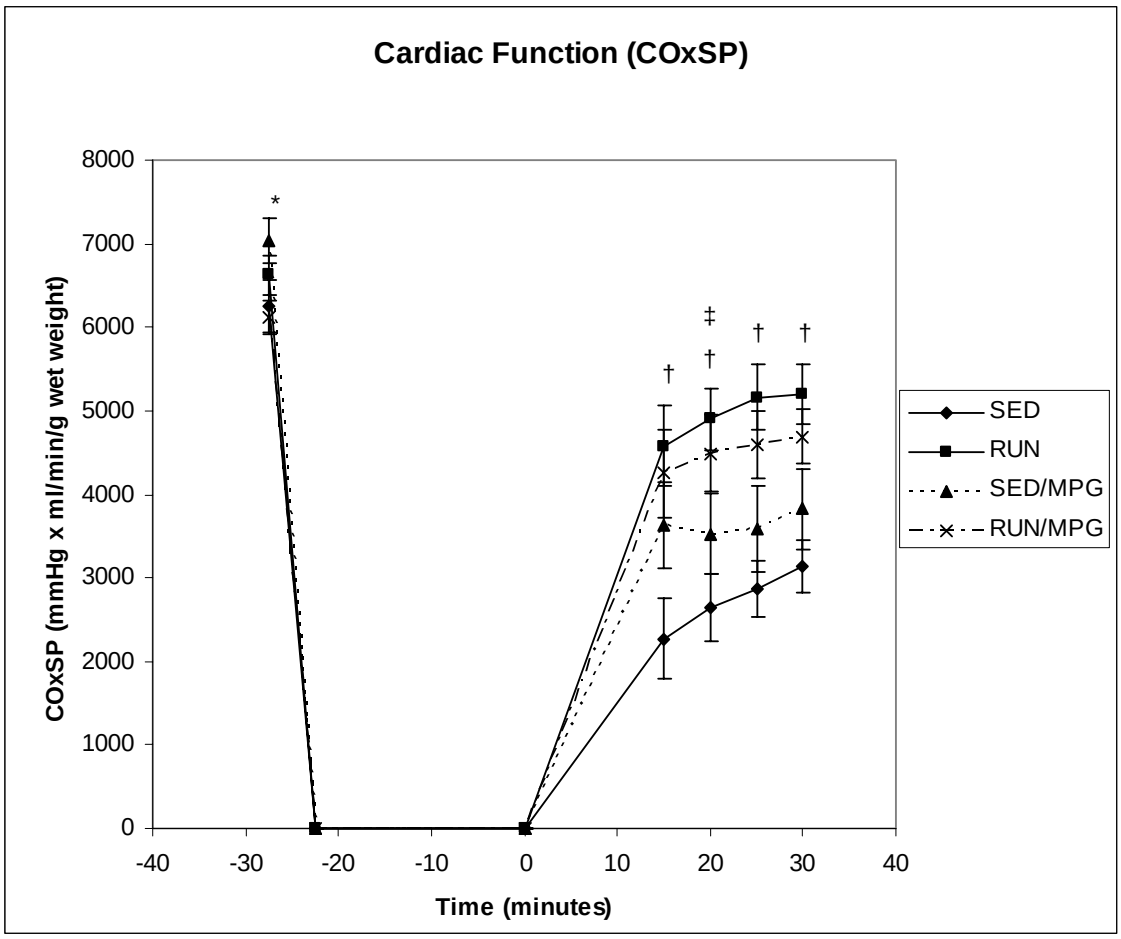
Lactate dehydrogenase (LDH) release in the coronary effluent over time, in the absence of ischemia. Values are mean ± SE, n=6. *($P<0.05$) vs. 10 minutes.

FIGURE 5 – CARDIAC EFFICIENCY, QUESTION 1



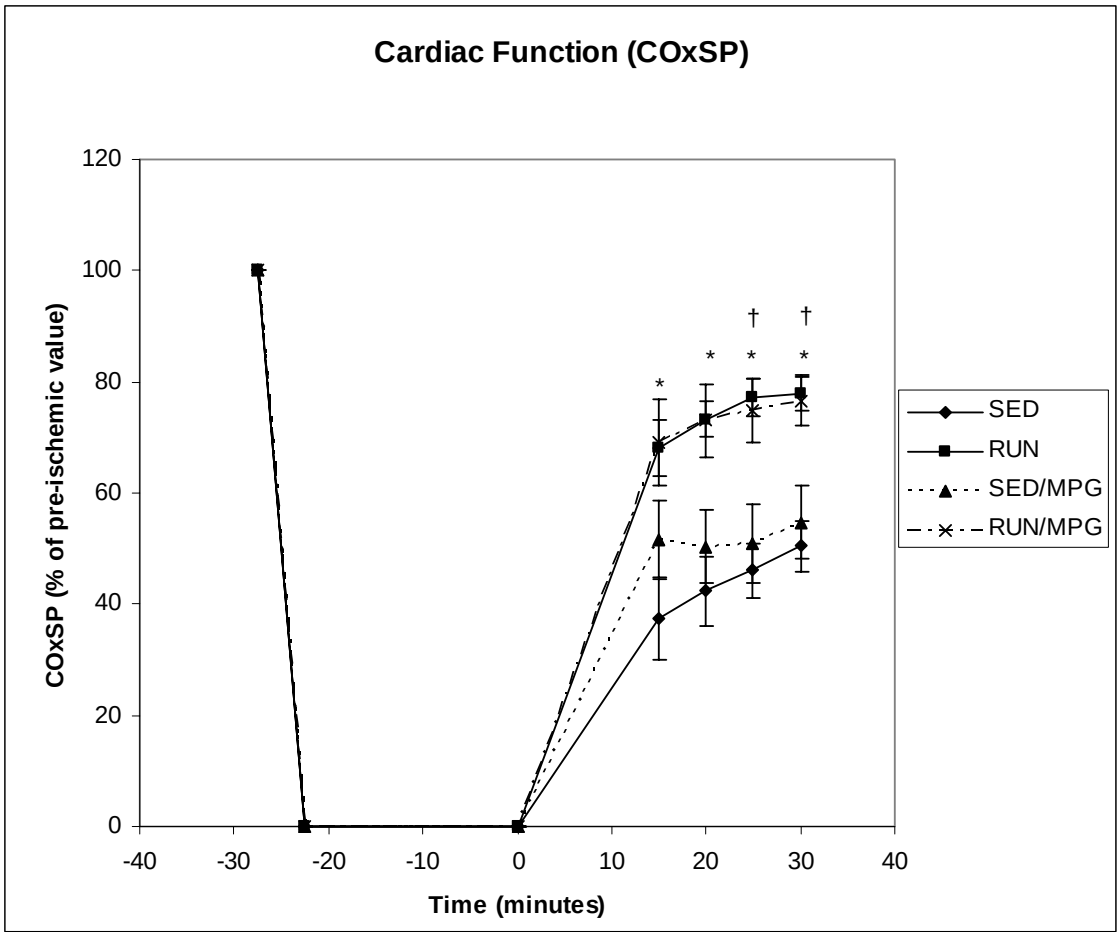
Cardiac efficiency (COxSP/oxygen consumption). Values are mean ± SE. *($P<0.05$) RUN vs. SED.

FIGURE 6 – CARDIAC FUNCTION (ABSOLUTE), QUESTION 2



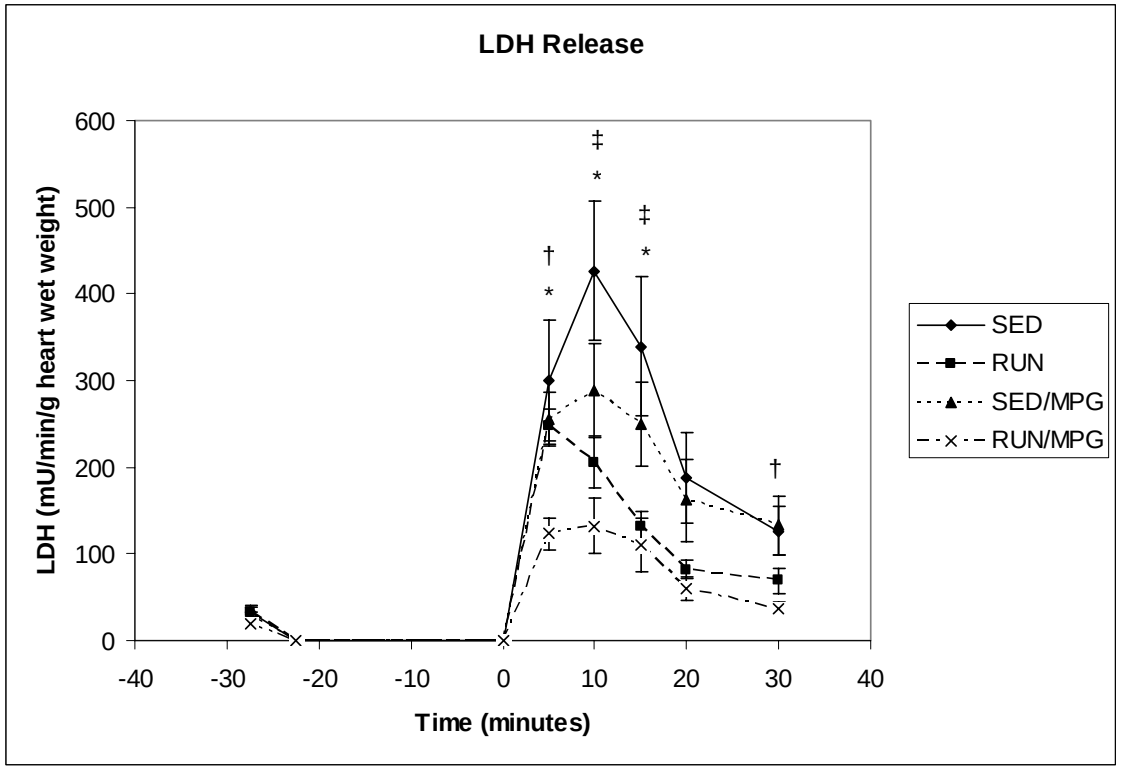
Cardiac function (COxSP), expressed as mmHG x mL/min/g heart wet weight. Values are mean ± SE. *($P<0.05$) SED/MPG vs. RUN/MPG, †($P<0.05$) SED vs. RUN, ‡($P<0.05$) SED vs. RUN/MPG.

FIGURE 7 – CARDIAC FUNCTION (PERCENTAGE), QUESTION 2



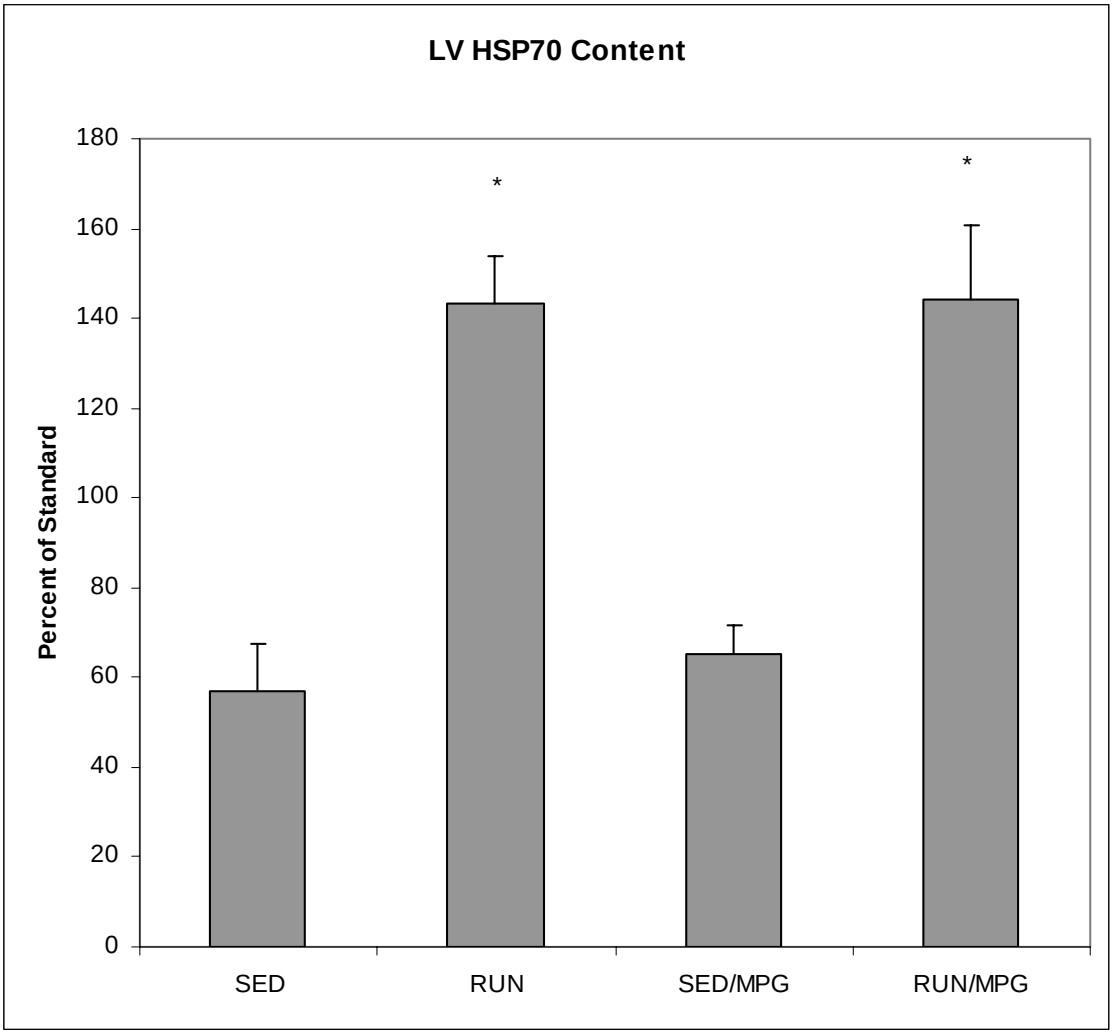
Cardiac function (COxSP), expressed as a percentage of pre-ischemic value. Values are mean ± SE. *(P<0.05) SED vs. RUN and RUN/MPG, †(P<0.05) SED/MPG vs. RUN and RUN/MPG.

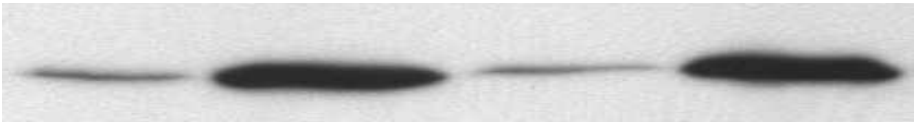
FIGURE 8 – LDH RELEASE, QUESTION 2



Lactate dehydrogenase (LDH) release in the coronary effluent. Values are mean ± SE. All groups are increased from pre-ischemic values 5, 10, and 15 minutes following ischemia ($P<0.05$). *($P<0.05$) SED vs. RUN/MPG, †($P<0.05$) SED/MPG vs. RUN/MPG; ‡($P<0.05$) SED vs. RUN.

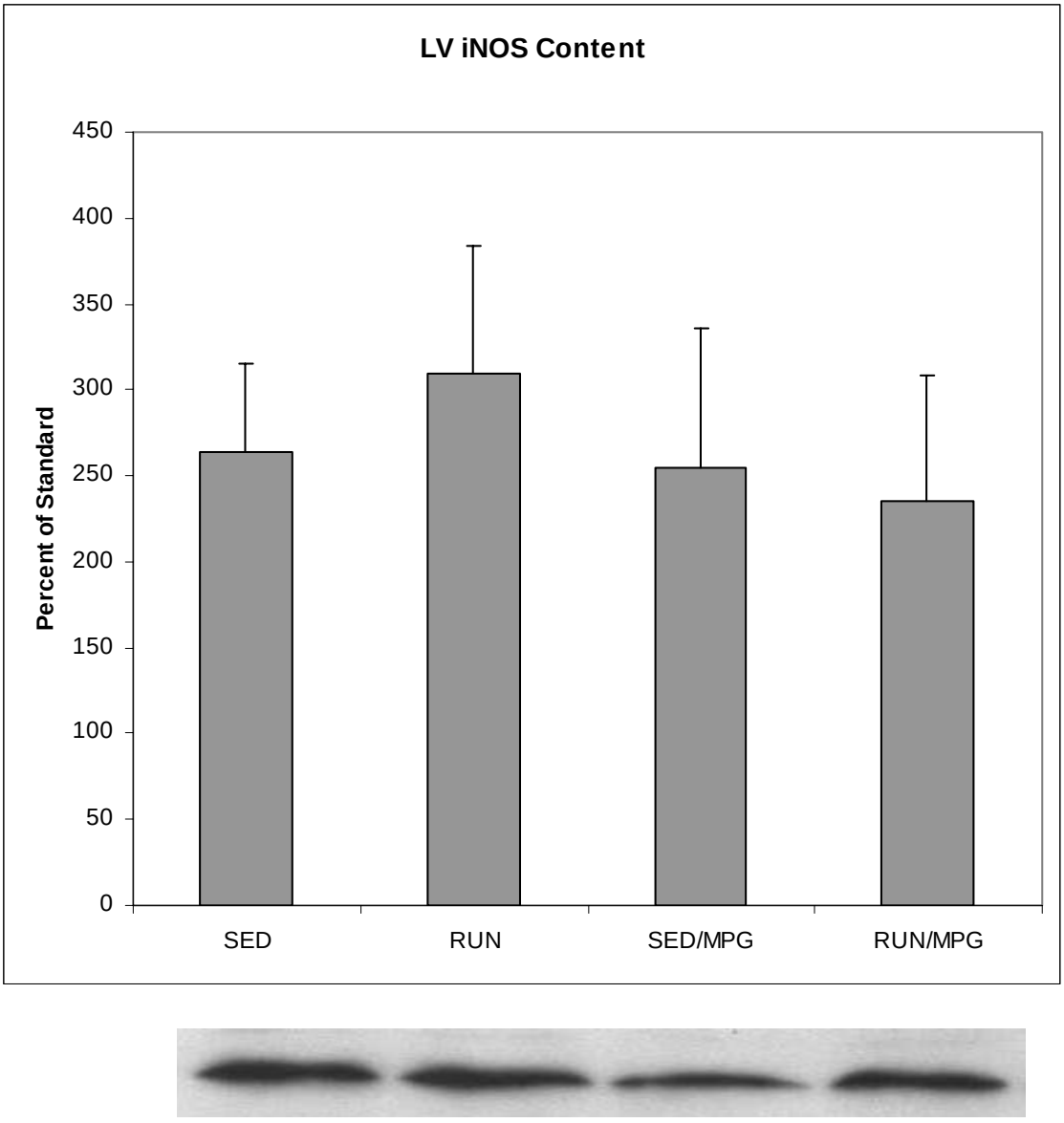
FIGURE 9 – HSP70 EXPRESSION, QUESTION 2





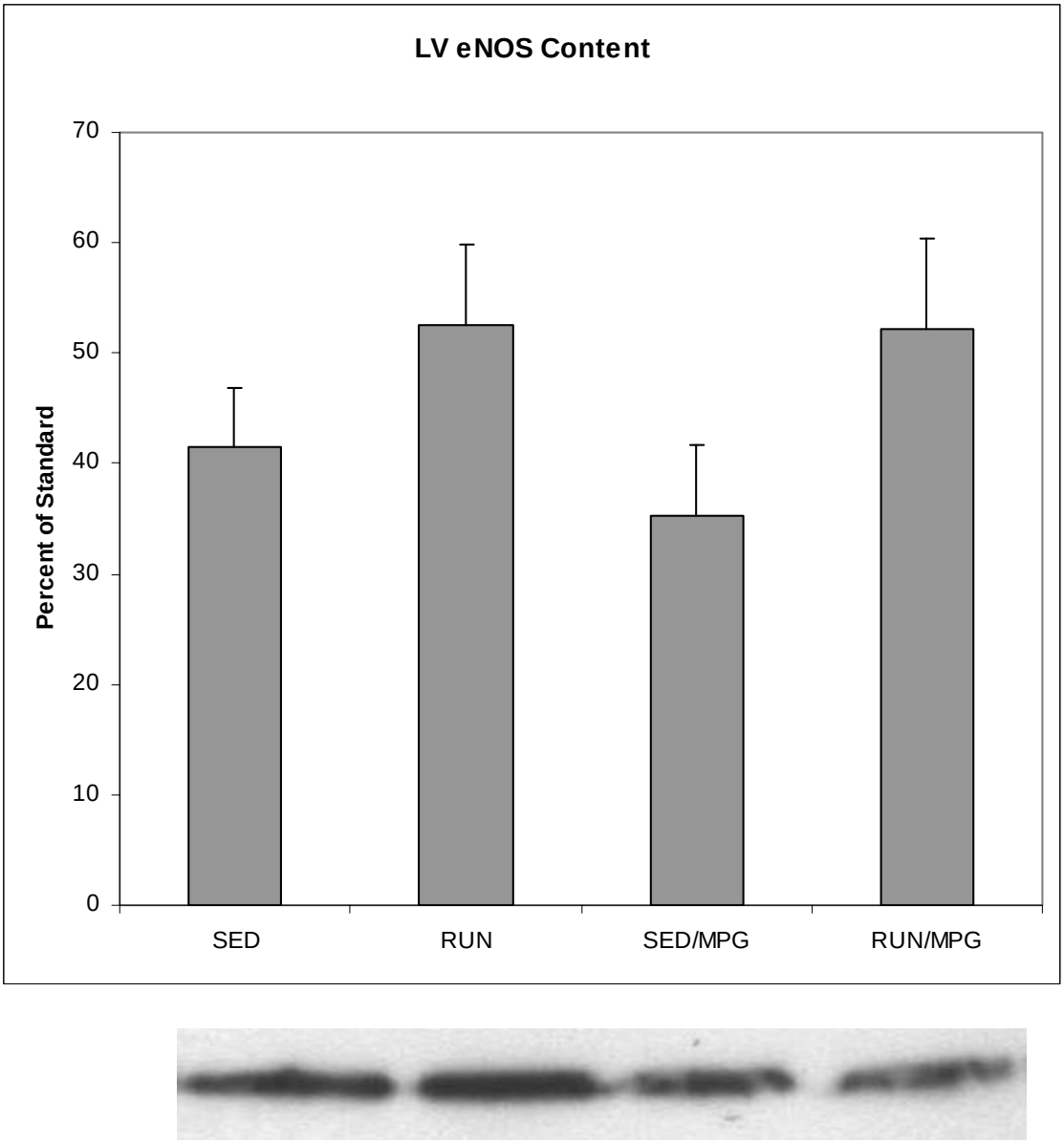
Heat shock protein 70 (HSP70) content of the left ventricle (LV), expressed as a percentage of a standard solution of HSP70. Values are mean ± SE. *($P<0.05$) RUN and RUN/MPG vs. SED and SED/MPG. Representative scan of western blot below figure.

FIGURE 10 – iNOS EXPRESSION, QUESTION 2



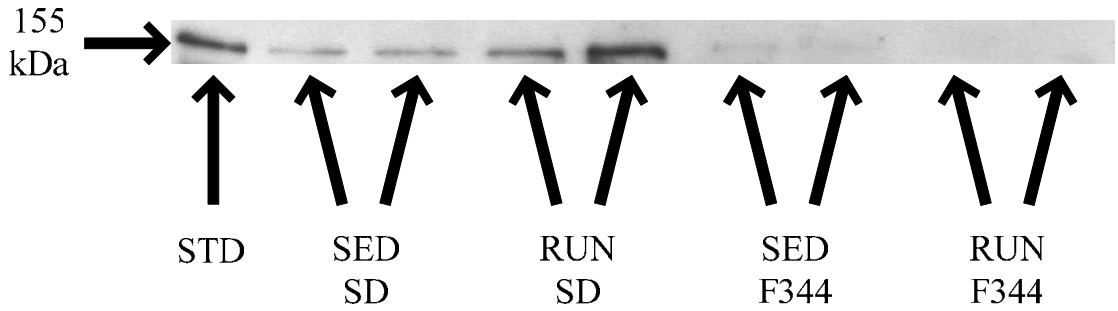
Inducible nitric oxide synthase (iNOS) content of the left ventricle (LV), expressed as a percentage of a standard solution of iNOS. Values are mean ± SE. No significant differences (P>0.05). Representative scan of western blot below figure.

FIGURE 11 – eNOS EXPRESSION, QUESTION 2



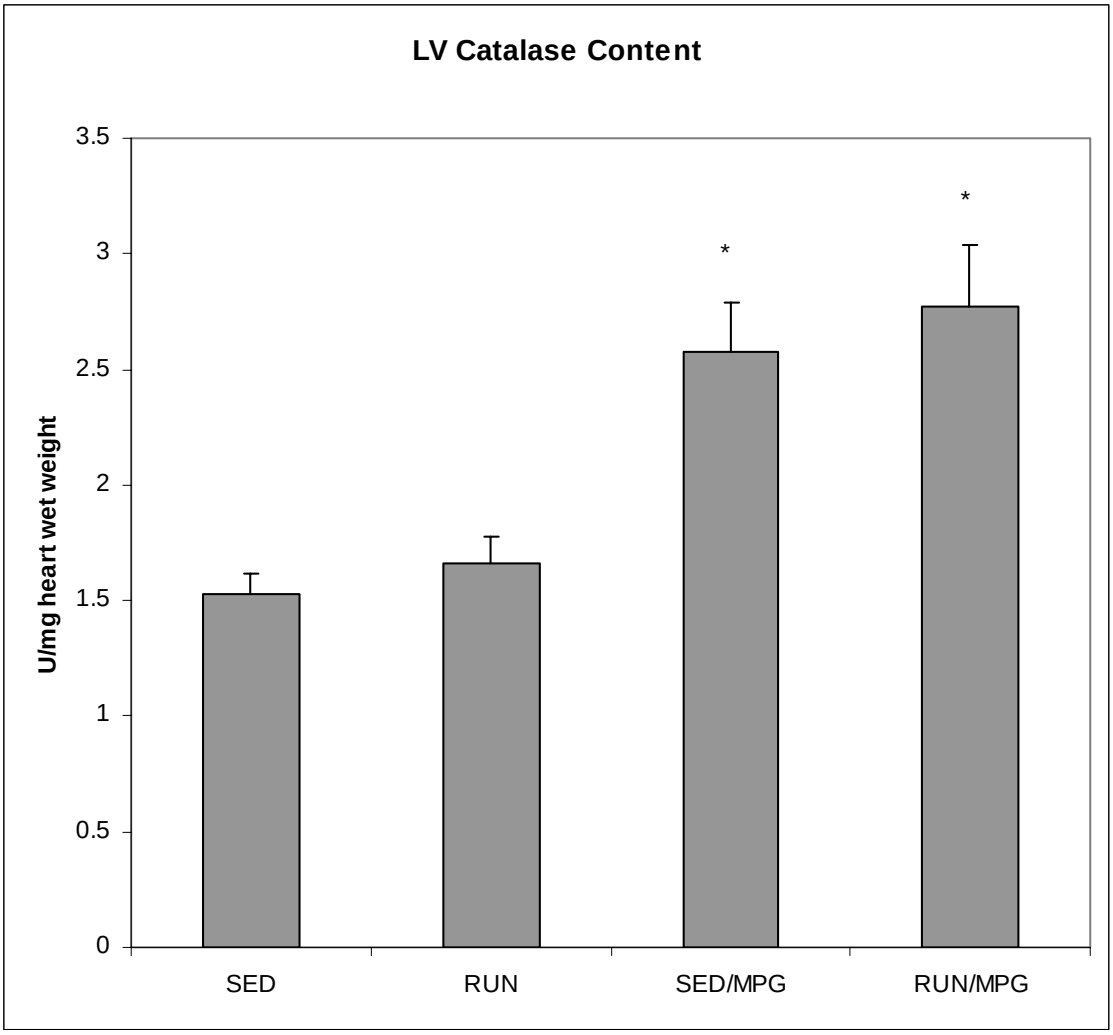
Endothelial nitric oxide synthase (eNOS) content of the left ventricle (LV), expressed as a percentage of a standard solution of eNOS. Values are mean ± SE. No significant differences (P>0.05). Representative scan of western blot below figure.

FIGURE 12 – NNOS EXPRESSION



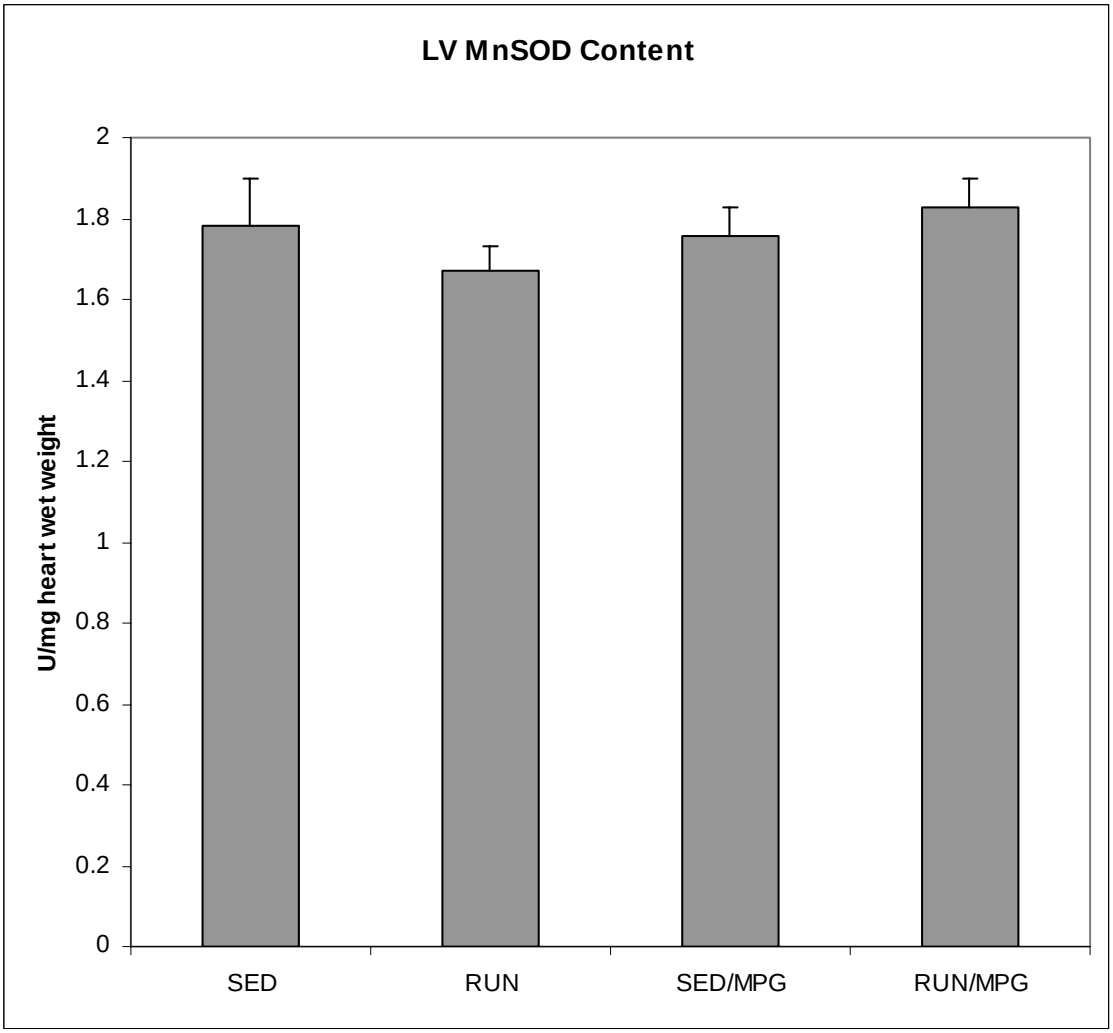
Comparison of neuronal nitric oxide synthase (nNOS) expression in the left ventricle of Sprague Dawley (SD) and Fischer 344 (F344) rats.

FIGURE 13 – CATALASE ACTIVITY, QUESTION 2



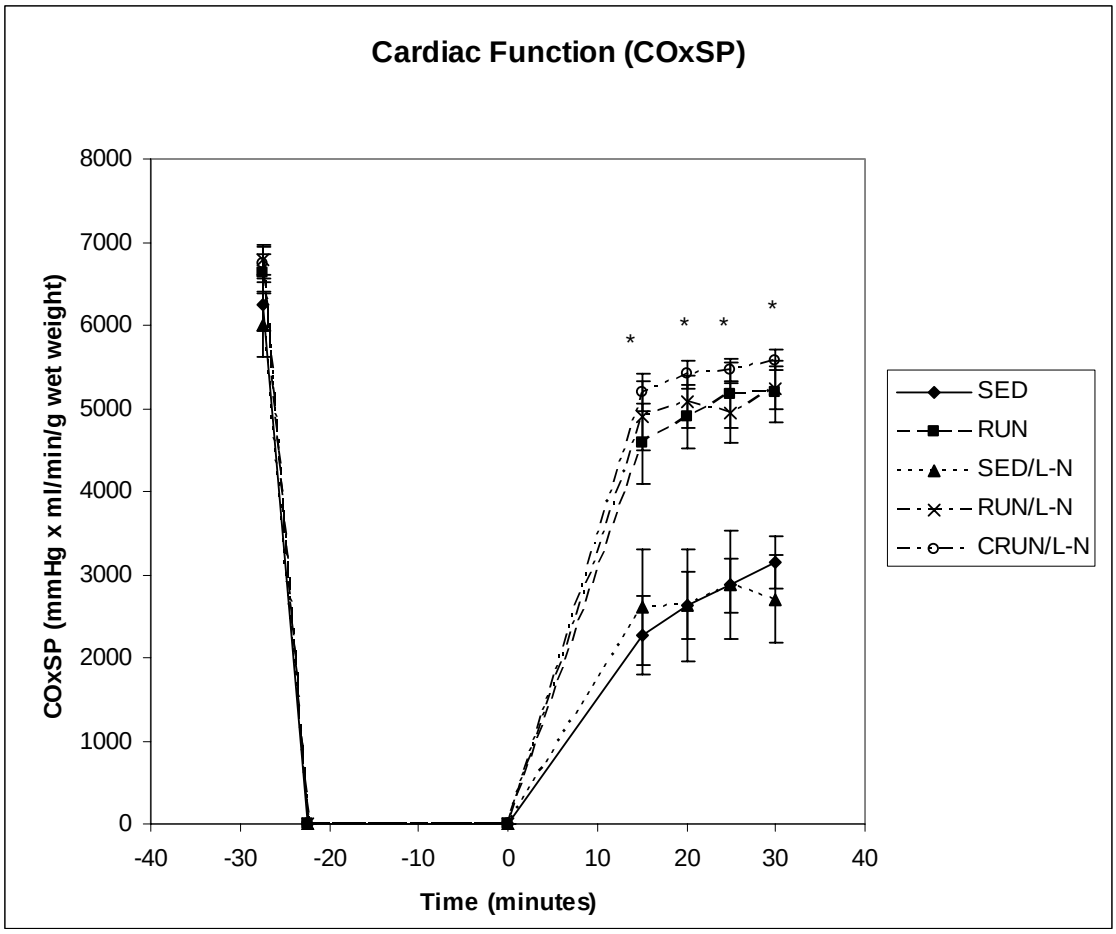
Enzymatic activity of catalase (CAT) in the left ventricle (LV), expressed as Units CAT/mg heart wet weight, where 1 Unit = 1 μmol H_2O_2/min. Values are mean ± SE. *($P<0.05$) SED/MPG and RUN/MPG vs. SED and RUN.

FIGURE 14 – MNSOD ACTIVITY, QUESTION 2



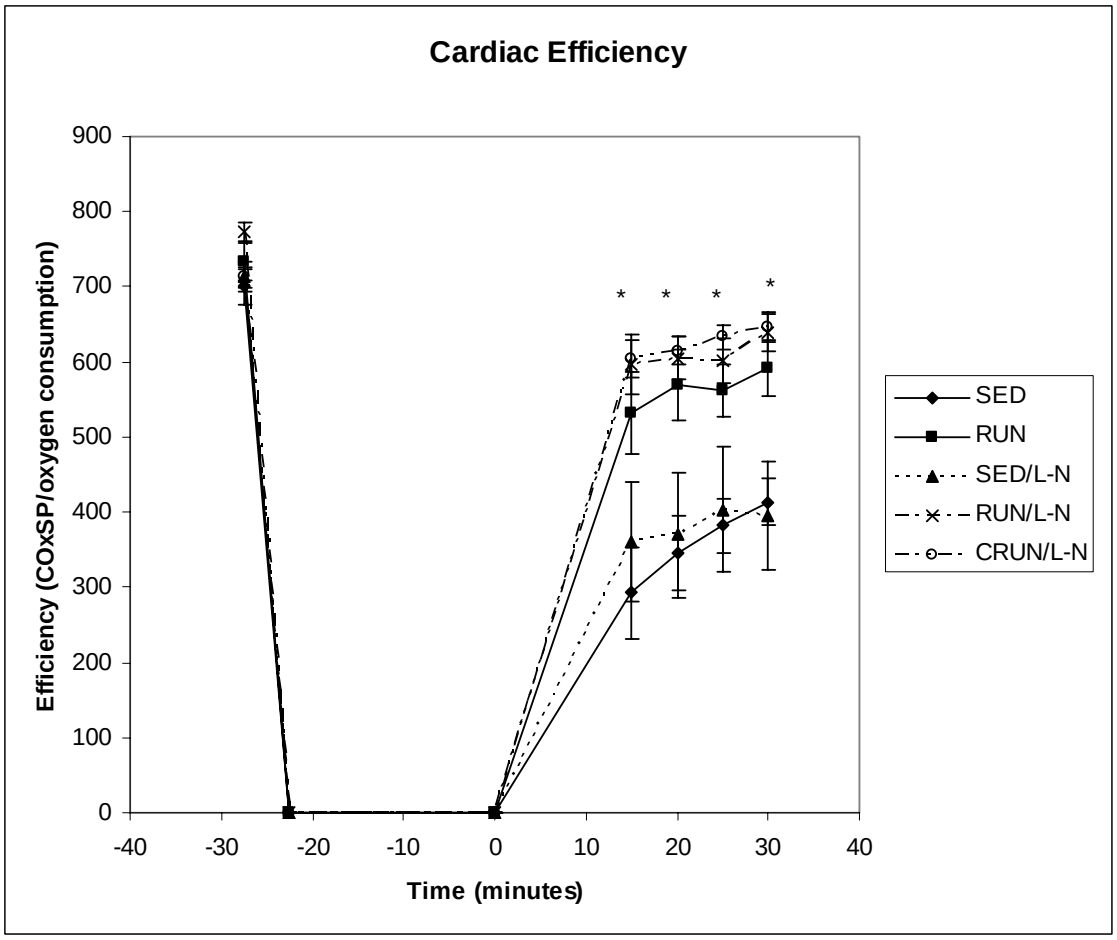
Enzymatic activity of manganese superoxide dismutase (MnSOD) in the left ventricle (LV), expressed as Units MnSOD/mg heart wet weight, where 1 unit = 50% inhibition of baseline. Values are mean ± SE. No significant differences (P>0.05).

FIGURE 15 – CARDIAC FUNCTION, QUESTION 4



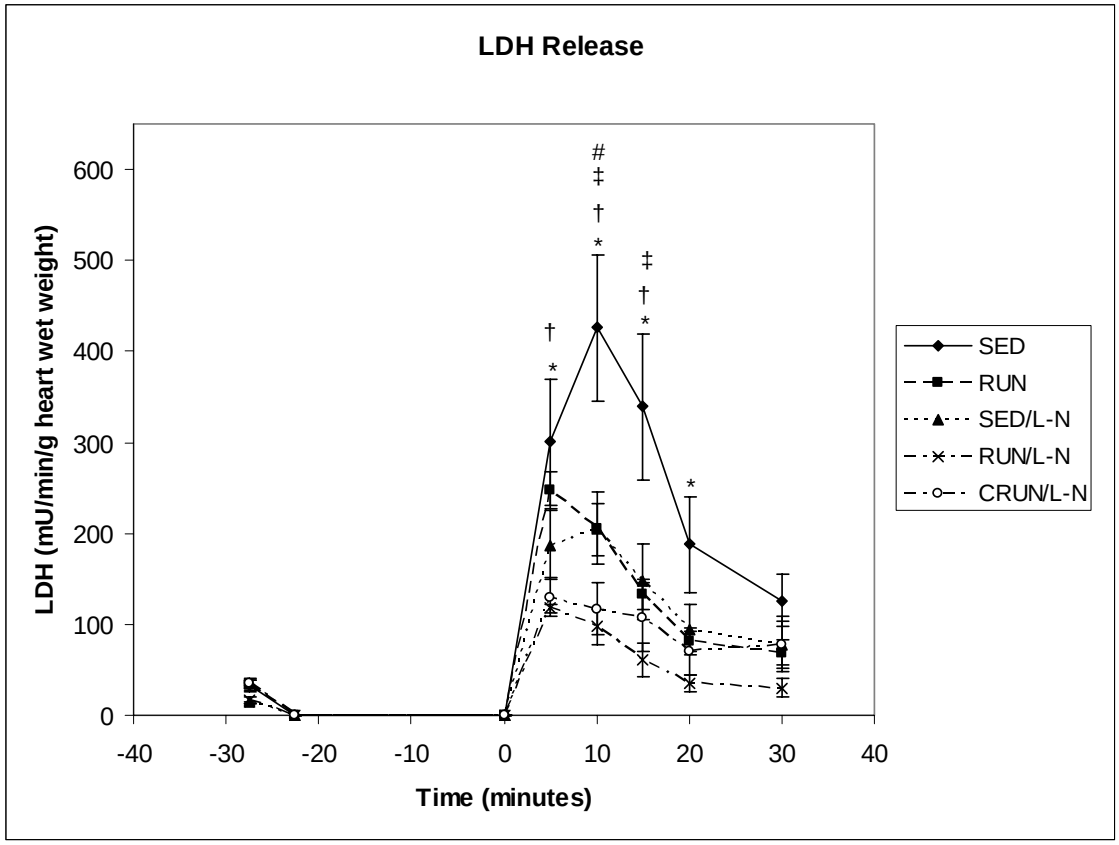
Cardiac function (COxSP), expressed as mmHG x mL/min/g heart wet weight. Values are mean ± SE. *($P<0.05$) RUN, RUN/L-N and CRUN/L-N vs. SED and SED/L-N.

FIGURE 16 – CARDIAC EFFICIENCY, QUESTION 4



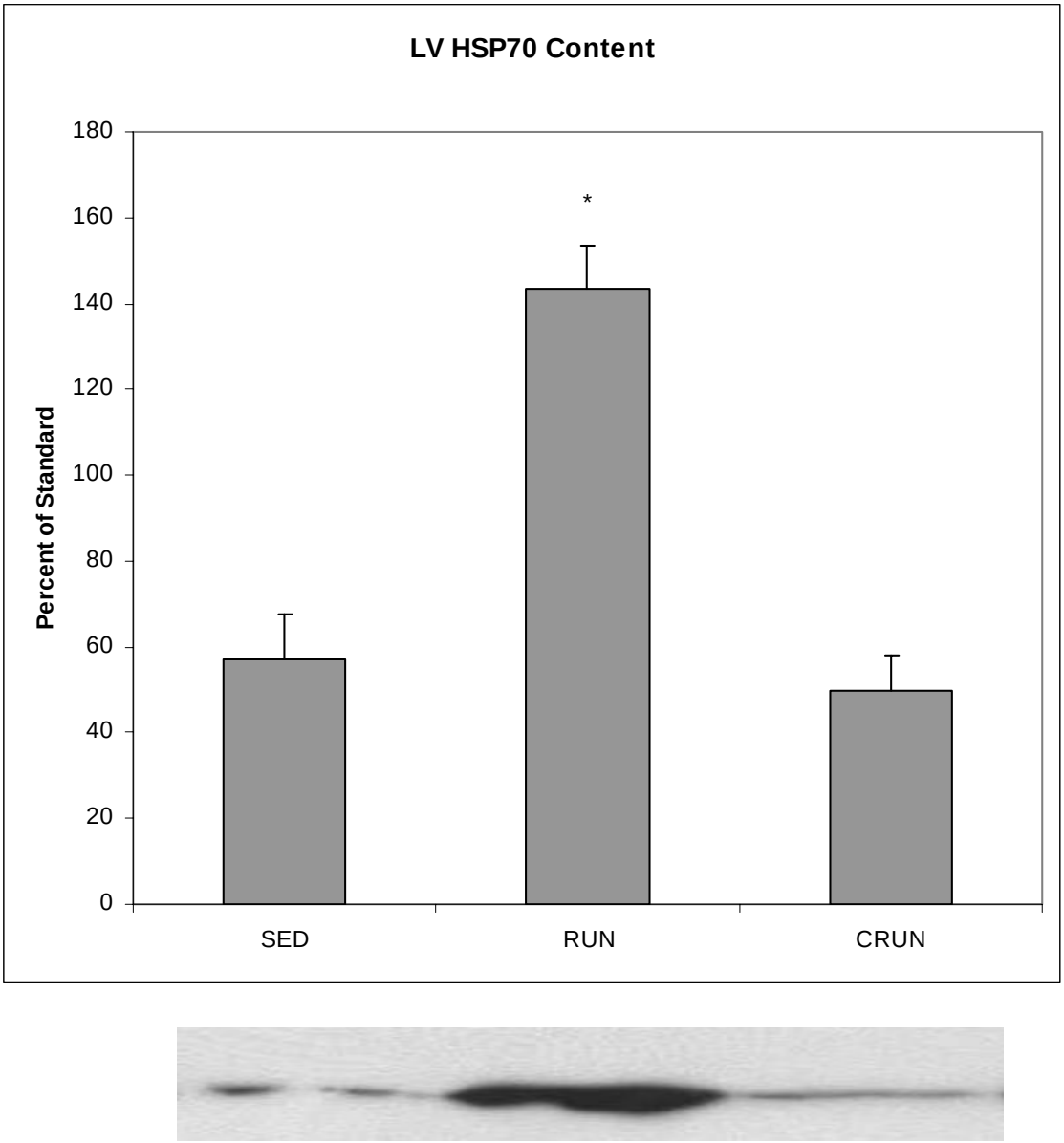
Cardiac efficiency (COxSP/oxygen consumption). Values are mean ± SE. *($P<0.05$) RUN, RUN/L-N, and CRUN/L-N vs. SED and SED/L-N.

FIGURE 17 – LDH RELEASE, QUESTION 4



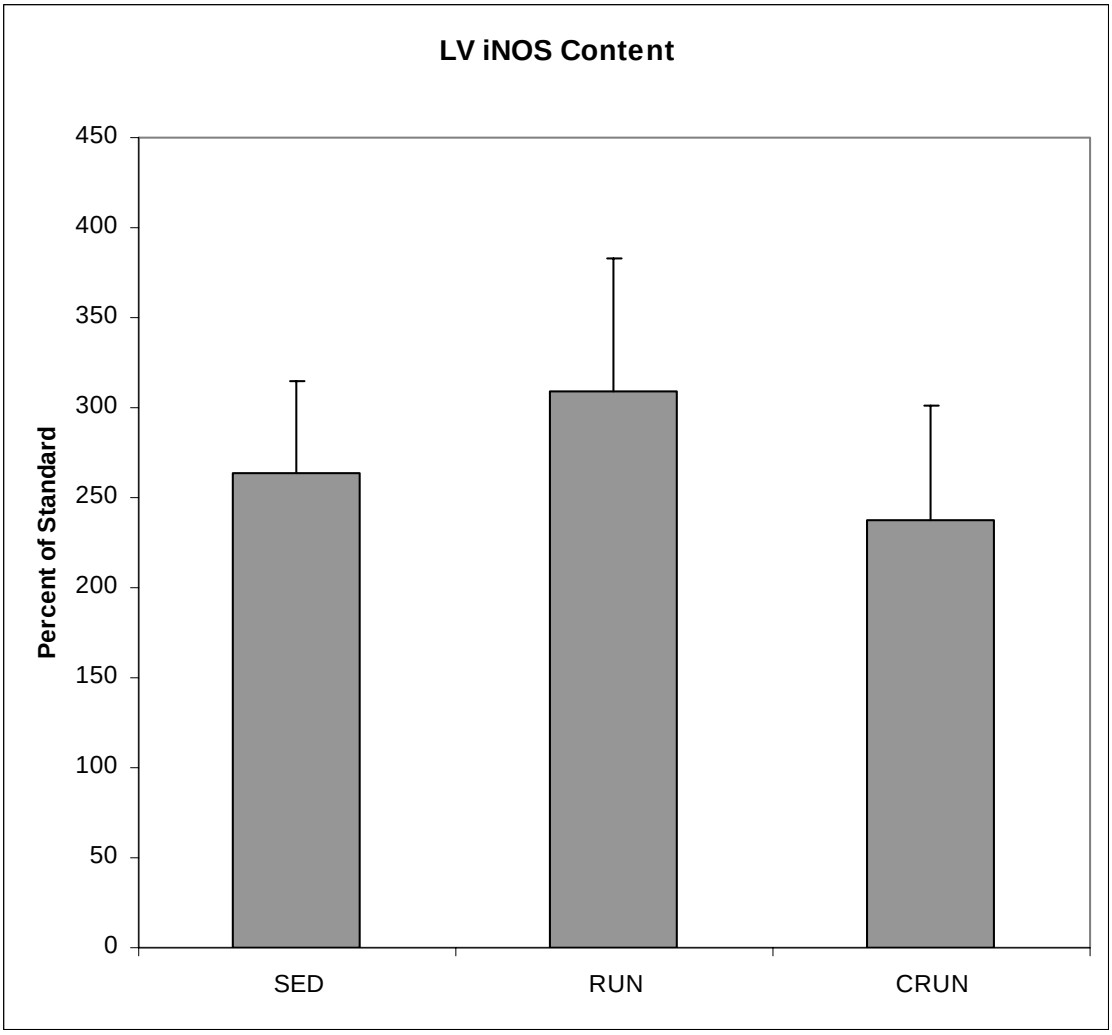
Lactate dehydrogenase (LDH) release in the coronary effluent. Values are mean ± SE. Groups SED/L-N and RUN/L-N increased from pre-ischemic values 5 and 10 minutes following ischemia ($P<0.05$) and no significant ($P>0.05$) increases were observed at any time point for CRUN/L-N. *($P<0.05$) SED vs. RUN/L-N, †($P<0.05$) SED vs. CRUN/L-N, ‡($P<0.05$) SED vs. RUN, #($P<0.05$) SED vs. SED/L-N.

FIGURE 18 – HSP70 EXPRESSION, QUESTION 4



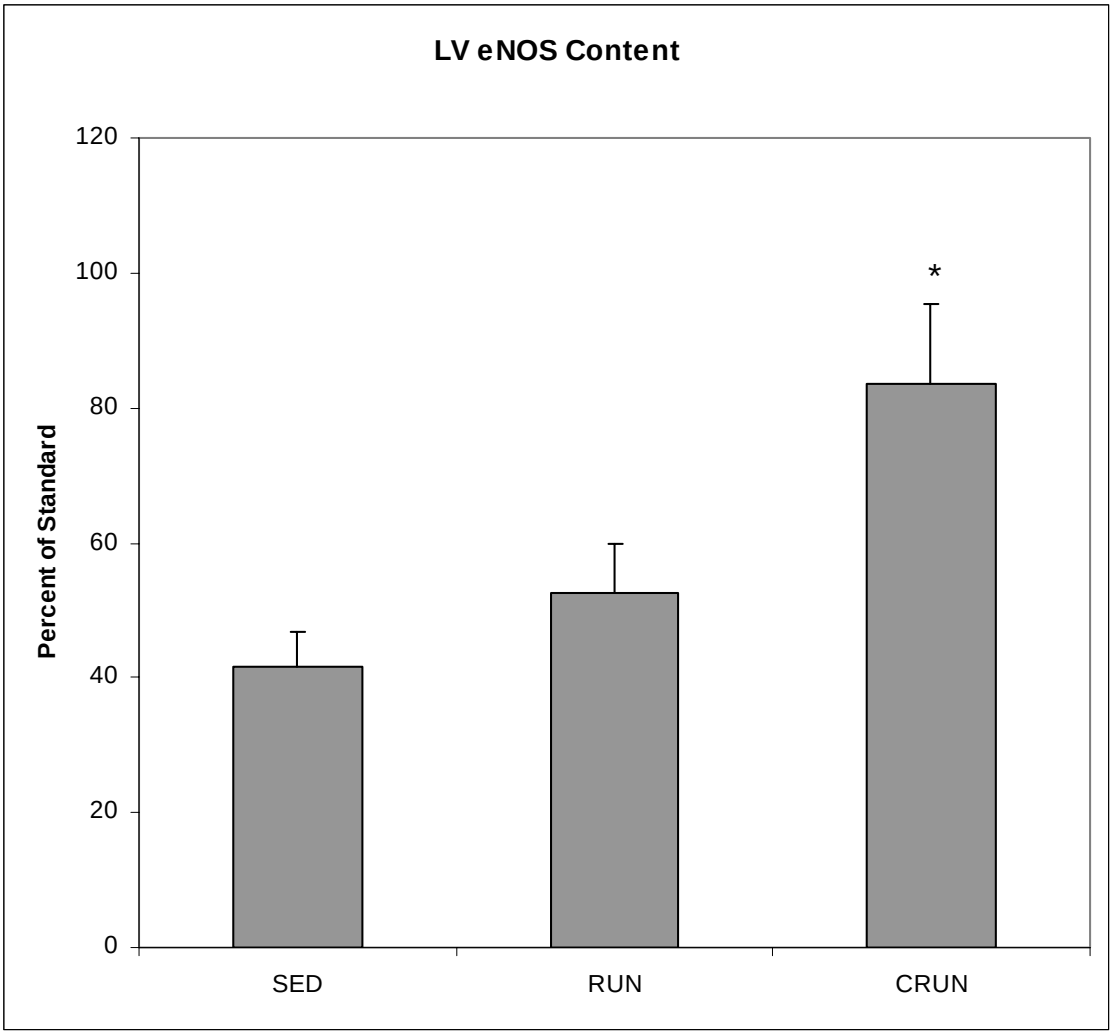
Heat shock protein 70 (HSP70) content of the left ventricle (LV), expressed as a percentage of a standard solution of HSP70. Values are mean ± SE. *($P<0.05$) RUN vs. SED and CRUN/L-N. Representative scan of western blot below figure.

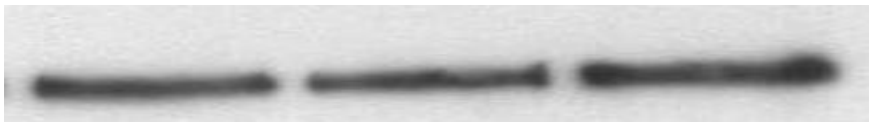
FIGURE 19 – iNOS EXPRESSION, QUESTION 4



Inducible nitric oxide synthase (iNOS) content of the left ventricle (LV), expressed as a percentage of a standard solution of iNOS. Values are mean ± SE. No significant differences (P>0.05). Representative scan of western blot below figure.

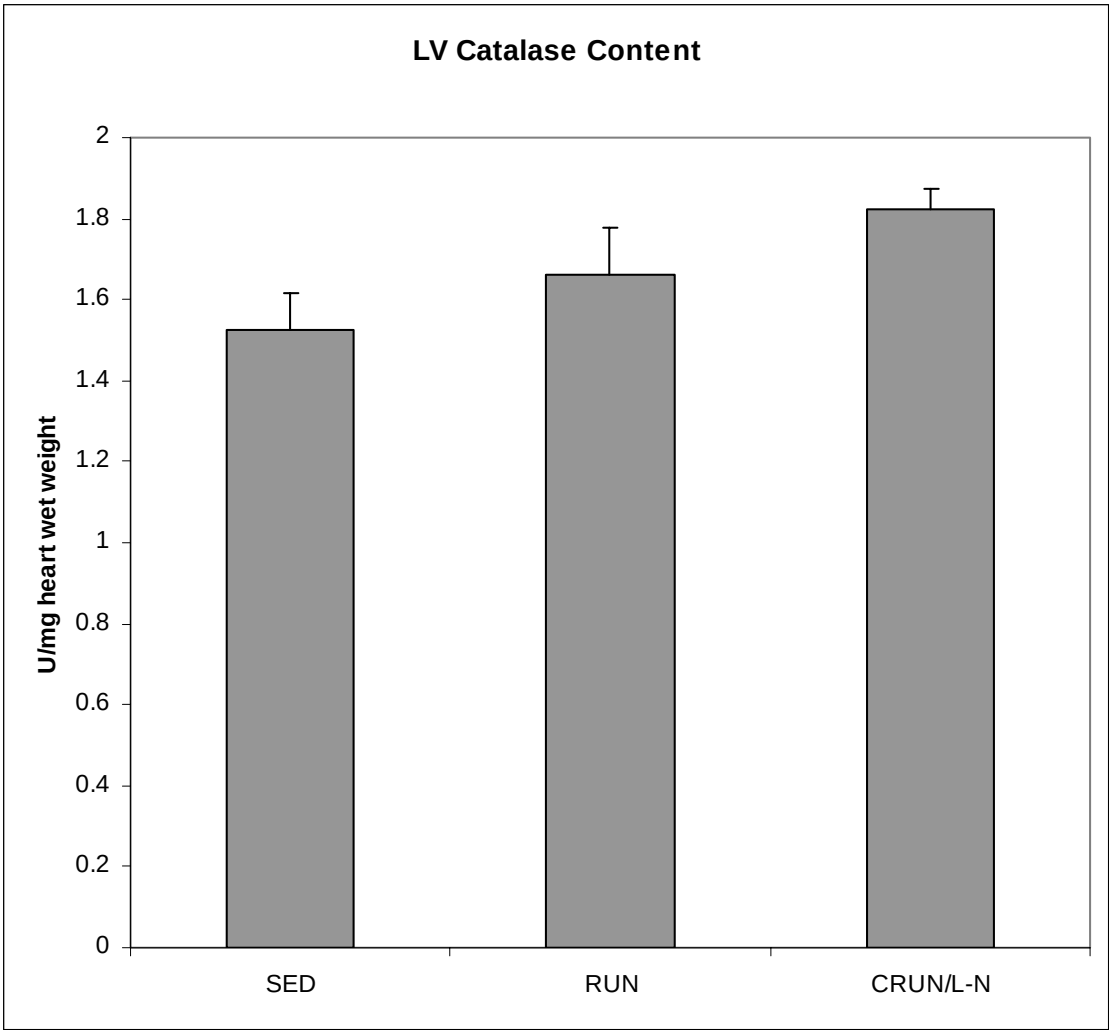
FIGURE 20 – eNOS EXPRESSION, QUESTION 4





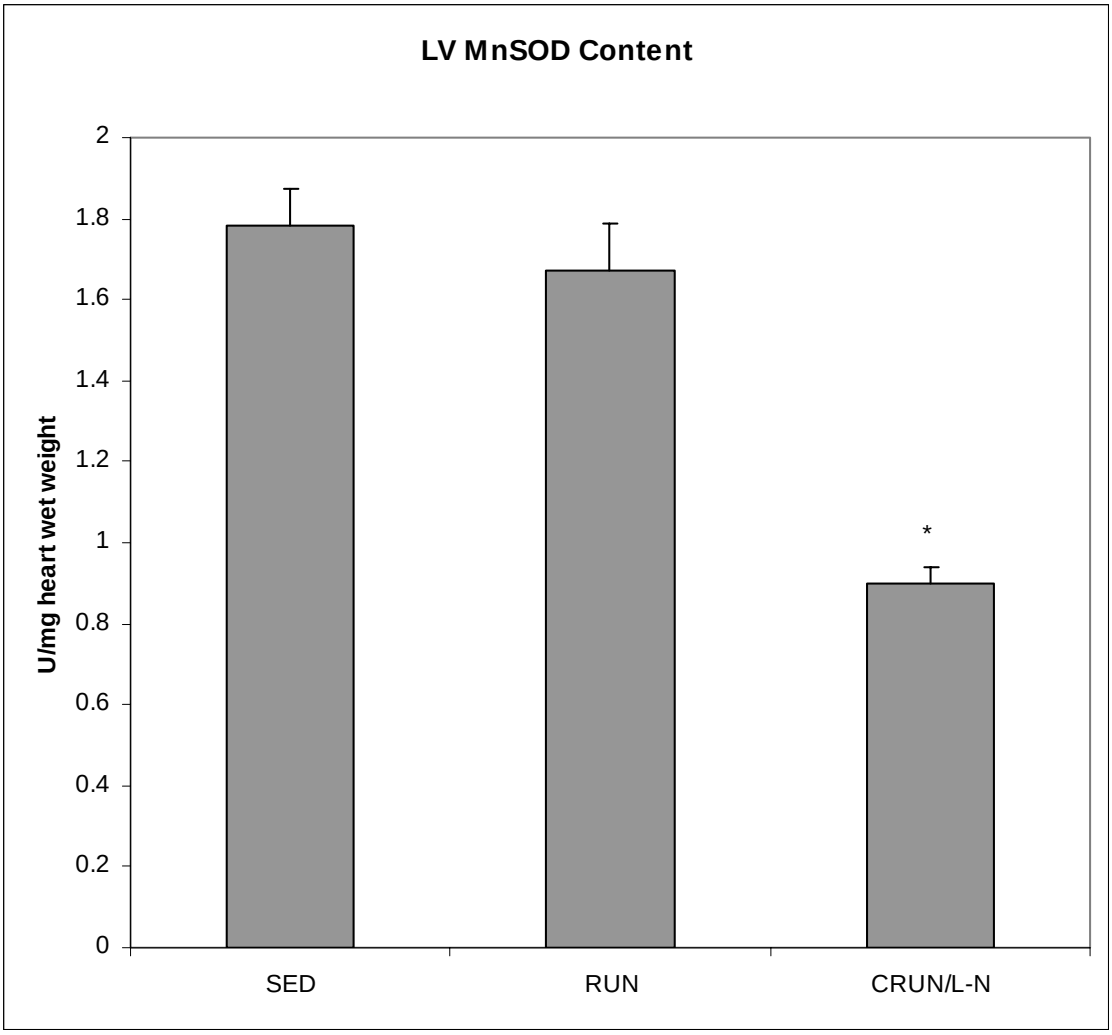
Endothelial nitric oxide synthase (eNOS) content of the left ventricle (LV), expressed as a percentage of a standard solution of eNOS. Values are mean ± SE. *($P<0.05$) CRUN/L-N vs. SED. Representative scan of western blot below figure.

FIGURE 21 – CATALASE ACTIVITY, QUESTION 4



Enzymatic activity of catalase (CAT) in the left ventricle (LV), expressed as Units/mg heart wet weight, where 1 Unit = 1 μmol H_2O_2/min. Values are mean ± SE. No significant differences (P>0.05).

FIGURE 22 – MNSOD ACTIVITY, QUESTION 4



Enzymatic activity of manganese superoxide dismutase (MnSOD) in the left ventricle (LV), expressed as Units MnSOD/mg heart wet weight, where 1 unit = 50% inhibition of baseline. Values are mean ± SE. *($P<0.05$) CRUN/L-N vs. SED and RUN.

Appendices

APPENDIX A- ANIMAL USE

1. *Description of use*. During the duration of this project, approximately 90 animals were utilized. Animals were purchased at 3 months of age and evaluated approximately 1-2 months after arrival. On the day of the experiment, rats were transported to the animal vivarium and environmental chamber located in Rm. 330, Bellmont Hall. 24 hours following experimental treatment, including treadmill running and/or intraperitoneal injection of physiological inhibitors, the rats were transported to the laboratory of Dr. J. W. Starnes located in Rm. 822A, Bellmont Hall. In Dr. Starnes' lab the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital at 40 mg/kg body weight, which will prevented any conscious response during surgery. The heart was then excised from the anesthetized animal and all experiments carried out on the isolated heart.
2. *Justification of use*. I have demonstrated that exercise training results in better recovery of myocardial contractile pump function following ischemia and subsequent reperfusion. This exercise-induced beneficial adaptation may be at least partially responsible for the increased survivability of heart attacks reported in physically active humans and the decreased risk of having a heart attack during physical activity in people who regularly exercise. This exercise-induced beneficial adaptation may also include protection against oxidative stress. The

overall specific aim of this research project was to determine the role of certain protective proteins in the attenuation of ischemia-reperfusion injury and protection against oxidative stress in the myocardium of acute exercise-trained rats. Finding ways to protect the heart during ischemic episodes is important as the occurrence of these episodes may lead to myocardial infarction, which is the leading cause of death in the United States. Human subjects are inappropriate because the end-point of the experiments requires that the heart be damaged. Non-animal species are inappropriate because they do not have a heart that is similar to that of a human. The rat is deemed most appropriate because of the prior studies carried out on this specie, ease of handling, and expense.

3. *Information on veterinary care*. Animals were housed in a central animal care facility under the direction of veterinarian specializing in laboratory animals. The University of Texas at Austin has an animal welfare assurance letter on file with NIH (#A 1496).
4. *Procedures for ensuring that discomfort, distress, pain and injury are limited and the use of anesthetic drugs*. There is modest risk of injury while exercising on the rodent treadmill. This risk was minimized by first acclimatizing the rats to the treadmill at sub-maximum intensities so that stress and fatigue was not a factor. If a rat refused to run, it was removed from the study. The only injury experienced by a rat was a cut toenail, and the animal was removed from the treadmill and antibiotic cream was administered to the toe area. The rats were anesthetized with sodium pentobarbital at a dosage of 40 mg/kg body weight,

administered intraperitoneally by Ryan Taylor who has been trained in this procedure by Dr. Joseph Starnes, who has been routinely carrying out this procedure since 1976.

5. *Method of euthanasia*. Removing the heart from the anesthetized animal produced a euthanizing effect. This method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Dead animals were placed in the morgue located at the Animal Resources Center.

APPENDIX B- KREBS-HENSELEIT BUFFER

Solutions:

3.85 M NaCl (58.4 g/mol) (Sigma # S-9625) +

0.325 M Glucose (180.2 g/mol) (Sigma # G-8270):

For 1000 mL

224.8 g NaCl + 58.6 g Glucose in dH_2O

1.54 M KCl (74.6 g/mol) (Sigma # P-4504):

For 500 mL

57.4 g in dH_2O

1.30 M $CaCl_2$ + 2 H_2O (147.0 g/mol) (Sigma # C-3881):

For 250 mL

47.8 g in dH_2O

1.56 M $MgSO_4$ + 7 H_2O (246.5 g/mol) (Sigma # M-1880):

For 250 mL

96.1 g in dH_2O

0.13 M Na_2 EDTA + 2 H_2O (372.2 g/mol) (Sigma # E-5134):

For 500 mL

24.2 g in dH_2O

*store all above solutions @ 4°C

25 U/mL Insulin (27 U/mg) (Sigma # I-5500):

For 50 mL

46.3 mg in 0.01 M HCl

*store @ -20°C

$NaHCO_3$ (84.0 g/mol) (Sigma # S-8875):

*Prepare Fresh Daily in dH_2O

Final Concentrations

118.5 mM	NaCl
10 mM	Glucose
4.7 mM	KCl
1.75 mM	$CaCl_2$
1.2 mM	$MgSO_4$
0.5 mM	Na_2 EDTA
12 mU/mL	Insulin
24.7 mM	$NaHCO_3$

Procedure:

Final Volume (L)	**1.30**	**1.95**	**3.25**	**4.55**	**5.85**
NaCl + Glucose	40	60	100	140	180
KCl	4	6	10	14	18
$CaCl_2$	1.75	2.62	4.37	6.13	7.88
$MgSO_4$	1	1.5	2.5	3.5	4.5
Na_2 EDTA	5	7.5	12.5	17.5	22.5

(All volumes in mL)

* Combine all of the solutions listed above, filter into flask, and follow with 1000 mL of dH_2O. For volumes listed below greater than initial 1000 mL, add additional volume of dH_2O to reservoir without filtering. Transfer filtered solution to reservoir and gas with 95:5 % O_2/CO_2 prior to adding $NaHCO_3$ solution.

dH_2O (mL)	1000	1500	2500	3500	4500

* Dissolve solid $NaHCO_3$ in 150 mL dH_2O, filter into flask, and follow with remaining volume of dH_2O.

$NaHCO_3$ (g)	2.70	4.05	6.75	9.45	12.15
dH_2O (mL)	247	371	617	863	1110

* After all solutions have been gassed, add appropriate volume of insulin without filtering.

25 U/mL Insulin (mL)	0.625	0.938	1.563	2.188	2.813

APPENDIX C- LDH ASSAY

Solutions:

Reaction buffer:

For 20 mL

Triethanolamine	0.473 M, pH 7.6	5.3 mL
NADH	30 mM	150 µL
Na_2EDTA	130 mM	240 µL
Pyruvate	1.0 M	50 µL
in dH_2O		

Procedure:

1. Collect samples of coronary flow during perfusion, and store in 4°C until analysis.
2. Add 250 µL of coronary effluent to 750 µL of reaction buffer in a 1.5 mL plastic cuvette.
3. Record Δ Abs @340 nm, between minutes 1 and 4 at 25°C.
4. LDH (mU/min/g) = Δ Abs x coronary flow rate (mL/min/g wet weight) x 788

APPENDIX D- HOMOGENIZATION PROTOCOL

Solutions:

Buffer:

50 mM K_2HPO_4, 0.1 mM EDTA, 0.1% TRITON X-100, pH 7.4

Procedure:

1. Trim approximately 150 mg of left ventricle from frozen heart.
2. Weigh tissue.
3. Calculate buffer volume as: mass of tissue in grams X 19 = volume in mL of sodium phosphate buffer.
4. Cut tissue into small pieces and place in homogenization tube.
5. Add volume of buffer from step 3 to homogenization tube, and place on ice.
6. Insert Teflon wand and start rotation of wand.
7. Homogenize by making approximately 50 passes of the wand into the tube.
8. Remove wand and inspect for non-homogenized pieces of tissue. Repeat additional passes if necessary.
9. Strain homogenate through one layer of cheese cloth.
10. Remove number of aliquots necessary for completion of assays and place remaining homogenate in -80°C.

APPENDIX E- LOWRY'S METHOD FOR DETERMINING PROTEIN CONCENTRATION

Solutions:

Protein Standard:

For 10 mL of 1 mg/mL stock:

10 mg Bovine Serum Albumin (Sigma # A-4378) in dH_2O.

Solution A:

For 2000 mL

40 g Na_2CO_3, 8 g NaOH, and 0.4 g NaK tartrate in dH_2O.

(Store in dark)

Solution B:

For 1000 mL

5 g $CuSO_4$, $5H_2O$ in dH_2O. (Store in the dark)

Solution C:

50 parts Solution A to 1 part Solution B. (must be prepared fresh)

Folin Ciocalteau's Phenol Reagent (Sigma # F-9252):

Dilute 5 parts of reagent with 6 parts dH_2O.

Procedure:

1. Standard Curve

   Prepare duplicate samples in the following manner:

[mg/mL]	1 mg/mL BSA stock (µL)	dH_2O (µL)	Final Volume (µL)
0.00	0	500	500
0.03	15	485	500
0.06	30	470	500
0.09	45	455	500
0.12	60	440	500
0.15	75	425	500
0.18	90	410	500
0.21	105	395	500

   * note concentrations at 0.18 and 0.21 mg/mL are probably out of range, make sure std curve does not begin to level off if using these concentrations.

2. Experimental Samples, Each sample is done in duplicate. Add 490 µL of dH_2O, 10 µL of sample, and 2.5 mL of Solution C.

3. Initiation of reaction and reading, add 0.5 mL of diluted Folin Ciocalteau's Phenol Reagent to each sample and vortex. Incubate at room temperature for 30 minutes, with timing starting upon the first addition of the reagent. Read O.D. at 660 nm.

APPENDIX F- AFFINITY CHROMATOGRAPHY PROTOCOL

Solutions:

Buffer:

50 mM K_2HPO_4, 0.1 mM EDTA, 0.1% TRITON X-100, pH 7.4.

Sepharose:

2'5' ADP Sepharose (Amersham # 17070001).

10X PBS (phosphate buffered saline):

For 1000 mL

80 g NaCl, 2 g KCl, 14 g Na_2HPO_4 + 7 H_2O, 2.4 g KH_2PO_4, pH 7.4, in dH2O.

1X PBS:

For 1000 mL

100 mL of 10X PBS, in dH_2O.

Procedure:

1. Place entire contents of sepharose bottle (5 g) into a 50 mL centrifuge tube, and wash three times with buffer according to manufacturer's instructions.
2. Measure volume of washed beads, and add an equivalent volume of buffer to make a 50% sepharose slurry.
3. Spin 1 mL of the 1:20 tissue homogenate at 9,000 x g, for 10 minutes. Aspirate 850 µL supernatant and retain.
4. Add 100 µL of sepharose slurry to supernatant and incubate overnight at 4°C on orbital shaker.

5. Spin samples at 5,000 x g for 5 minutes Aspirate 800 µL and retain pellet.
6. Wash pellet 1 time x 5 minutes with 800 µL 1X PBS at 5,000 x g.
7. Resuspend pellet in 50 µL of 2X sample buffer, boil for 5 minutes, and centrifuge at 8,000 x g for 5 minutes.
8. Load 20 µL of supernatant onto gel, blot as described in western blotting optimization, Appendix I.

APPENDIX G- LAEMMLI SDS-PAGE (SODIUM DODECYL SULFATE – POLYACRYLAMIDE GEL ELECTROPHORESIS) PROCEDURE

Solutions:

40% acrylamide:

Stock solution (Bio-Rad # 161-0140)

2% bis-acrylamide:

Stock solution (Bio-Rad # 161-0142)

1.5 M Trizma:

For 500 mL

90.83 g Trizma Base in dH_2O, pH 6.8.

1.25 M Trizma:

For 500 mL

75.63 g Trizma Base in dH_2O, pH 6.8.

20% sodium dodecyl sulfate (20% SDS):

Stock solution (Bio-Rad # 161-0418)

TEMED:

Stock Solution of N, N, N', N' - tetramethylethylenediamine (Sigma # 87689)

ammonium persulfate (APS):

For 1 mL of 10%

100 mg APS in dH_2O. Prepared fresh daily.

10X Running Buffer:

For 1000 mL

30.28 g Trizma

144.2 g glycine

in dH_2O

1X Running Buffer:

For 1000 mL

100 mL 10X Running Buffer

5 mL 20 % SDS

in dH_2O

dithiothreitol (DTT):

For 10 mL of 2 M

3.086 g in dH_2O.

2X Sample Buffer:

	2X	Final 1X Concentration
1.25 M Trizma, pH 6.8	5.0 mL	62.5 mM
Glycerol	10.0 mL	10.0%
20% SDS	5.0 mL	1.0%
bromophenol blue	0.004 g	0.004%
dH_2O	25.0 mL	

To use sample buffer, add 100 μL of 2 M DTT to 0.9 mL of 2X sample buffer.

Resolving Gel 12.5%:

	For every 2 gels
40% acrylamide	3.15 mL
2% bis-acrylamide	0.35 mL
1.5 M Trizma	2.5 mL
20% SDS	50 µL
dH_2O	3.95 mL
10% APS	32.5 µL
TEMED	6.25 µL

Add 10% APS and TEMED added prior to casting (Begins polymerization).

Stacking Gel 5%:

	For every 2 gels
40% acrylamide	1.25 mL
2% bis-acrylamide	0.65 mL
1.25 M Trizma	1 mL
20% SDS	50 µL
dH_2O	6.99 mL
10% APS	50 µL
TEMED	10 µL

Add 10% APS and TEMED added prior to casting (Begins polymerization).

Procedure:

1. Assemble gel apparatus according to manufacturer's instructions in casting stand.

   Prepare resolving gel solution for number of gels to be cast.

2. Gently swirl solution and then use a Pasteur pipette to fill caster 3/4 full.

   Overlay resolving gel with 200 µL of butanol and allow 1 hour for gel to polymerize.

3. Pour butanol off the resolving gel and rinse with dH_2O.

4. Prepare stacking gel solution for the number of gels cast, gently swirl solution, pour on top of the resolving gel and insert Teflon comb while avoiding bubbles. Allow 1 hour for polymerization.
5. While the stacking gel is polymerizing prepare samples. Samples that were homogenized 1:20 in sodium phosphate buffer are further diluted 1:2 with 2X sample buffer (100 µL sample: 100 µL 2X sample buffer) and allow to incubate 20 minutes at room temperature. Samples that were prepared through affinity chromatography will have been prepared in 1X sample buffer. Place samples in boiling water for 3 minutes prior to loading.
6. Following polymerization of the stacking gel, assemble the gel apparatus for electrophoresis and place in the electrophoresis chamber. Fill the inner chamber with running buffer and bring the outer chamber to 1/4 full with running buffer.
7. Load tissue homogenates (approximately 30 µL for homogenized samples and 20 µL for affinity chromatography) and appropriate amounts of standard. Electrophorese at a constant 50 mA per 2 gels and a maximum of 200 V and 200 W until the tracking dye runs off the gel.

APPENDIX H- WESTERN BLOTTING – SEMI-DRY TRANSFER

Solutions:

1 M Trizma:

For 1000 mL

121.1 g Trizma

in dH_2O

Anode 1 (for 1000 mL):

Reagent	Amount
300 mM Trizma	300 mL of 1 M Trizma
0.05% SDS	2.5 mL of 20% SDS
10% Methanol	100 mL
10 mM β-mercaptoethanol	0.78 mL

Add reagents to ~500 mL dH_2O and bring to volume.

Anode 2 (for 1000 mL):

Reagent	Amount
25 mM Trizma	25 mL of 1 M Trizma
0.05% SDS	2.5 mL of 20% SDS
10% Methanol	100 mL
10 mM β-mercaptoethanol	0.78 mL

Add reagents to ~500 mL dH_2O and bring to volume.

Cathode (for 1000 mL):

Reagent	Amount
25 mM Trizma	25 mL of 1 M Trizma
40 mM α-amino-hexanoic acid	5.248 g
0.05% SDS	2.5 mL of 20% SDS
10% Methanol	100 mL
10 mM β-mercaptoethanol	0.78 mL

Add reagents to ~500 mL dH_2O and bring to volume.

Procedure:

1. During the SDS-PAGE procedure, pre-wet the PVDF membranes in 100% methanol and then soak in anode 1 for a minimum of 20 minutes. (Use one PVDF membrane per gel, cut to the same dimensions as gel 5x10 cm for Bio-Rad mini-gel).
2. Once SDS-PAGE is completed, remove the resolving gel and soak in anode 1 for a minimum of 5 minutes.
3. Wet two sheets of filter paper in anode 2. Place the 2 sheets of filter paper on the Bio-Rad Trans-Blot electrode. Roll a test tube over the top of each piece to remove air bubbles.
4. Remove the PVDF membrane form anode 1 and place on top of the filter paper, remove air bubbles with test tube.
5. Remove the gel from anode 1, place on top of the PVDF membrane, and remove air bubbles with gloved finger.
6. Wet two sheets of filter paper in cathode, stack the sheets on top of the gel, and remove air bubbles with test tube.
7. Transfer the proteins from the gel to the PVDF membrane at a constant 150 mA, 15 V, and a maximum of 200 W for 35 minutes.
8. Following the transfer, place the PVDF membrane in dH_2O and refrigerate until immunoblotting is performed.

APPENDIX I- WESTERN BLOTTING - IMMUNOBLOTTING

Solutions:

10X PBS (phosphate buffered saline):

For 1000 mL

80 g NaCl

2 g KCL

14 g Na_2HPO_4 + 7 H_2O

2.4 g KH_2PO_4

pH 7.4 in dH_2O

1X PBS-.1% Tween:

For 1000 mL

100 mL 10X PBS

1 mL of Tween 20

in dH_2O

Primary antibody:

Dilute primary antibody to optimal concentration (Appendix J) in 1XPBS-Tween (10 mL per gel) with 1% milk.

Secondary antibody:

Dilute appropriate HRP-labeled secondary antibody to optimized concentration (Appendix J) in 1XPBS-Tween (10 mL per gel).

Procedure:

1. Block PVDF membranes for 1 hour at room temperature or overnight at 4°C in 1X PBS with 5% milk.
2. Rinse the membranes with two quick washes of 1XPBS-Tween.
3. Incubate membranes with primary antibody for 1 hour at room temperature on orbital shaker.
4. Wash 3X10 minutes in 1XPBS-Tween.
5. Incubate 1 hour with secondary antibody.
6. Wash 5X5 minutes with 1XPBS-Tween.
7. Rinse 3 times with dH_2O.
8. Prepare the membranes for autoradiography using PIERCE SuperSignal solutions (Pierce Chemical, Rockford, IL) according to manufacturer's instructions.
9. Expose membranes to Kodak Biomax film (no. Z370398, Sigma Aldrich Inc., St Louis, MO) for previously determined optimal exposure time.
10. Develop film according to manufacturer's instructions (Kodak autoradiography processing chemicals, no. p7042 & p7167, Sigma Aldrich Inc., St Louis, MO).
11. Images are then scanned into an Apple Power PC computer, and the density of the developed bands are quantified using NIH image software (NIH, Bethesda, MD).

APPENDIX J- BLOTTING OPTIMIZATION

HSP70:

Primary antibody

HSP70 (W27) mouse monoclonal IgG (no. sc-24, Santa Cruz Biotechnology) diluted 1:200.

Secondary antibody

Anti-mouse IgG HRP (horseradish peroxidase-linked whole antibody from sheep; no. NXA 931, Amersham Life Sciences) diluted 1:2500.

UCP2:

Primary antibody

UCP2 (C-20) goat polyclonal IgG (no. sc-6525, Santa Cruz Biotechnology) diluted 1:100.

Secondary antibody

Anti-goat IgG HRP (horseradish peroxidase-linked whole antibody from rabbit; no. sc-2768, Santa Cruz Biotechnology) diluted 1:20,000.

eNOS:

Primary antibody

eNOS mAb (no. 610297, BD Biosciences, San Diego, CA) diluted 1:200.

Secondary antibody

Anti-mouse IgG HRP (horseradish peroxidase-linked whole antibody from sheep; no. NXA 931, Amersham Life Sciences) diluted 1:2500.

iNOS:

Primary antibody

iNOS mAb (no. 610329, BD Biosciences, San Diego, CA) diluted 1:100.

Secondary antibody

Anti-mouse IgG HRP (horseradish peroxidase-linked whole antibody from sheep; no. NXA 931, Amersham Life Sciences) diluted 1:2000.

nNOS:

Primary antibody

nNOS mAb (no. 610309, BD Biosciences, San Diego, CA) diluted 1:100.

Secondary antibody

Anti-mouse IgG HRP (horseradish peroxidase-linked whole antibody from sheep; no. NXA 931, Amersham Life Sciences) diluted 1:2000.

APPENDIX K- CATALASE ASSAY

Sample Preparation:

1. Pipette 1 mL of tissue homogenate into centrifuge tube.
2. Centrifuge 1,500 x g for 10 minutes at 4°C, retain supernatant for analysis.

Solutions:

Buffer:

50 mM K_2HPO_4, 0.1 mM EDTA, 0.1% TRITON X-100, pH 7.4.

Hydrogen Peroxide:

1 M H_2O_2 stock solution

Procedure:

1. Equilibrate the incubation buffer with N_2 gas in order to decrease O_2 concentration to a very low amount so that the O_2 produced from the catalase reaction will remain in solution during the assay.
2. Add 1.5 mL nitrogen-equilibrated buffer to the oxygen electrode cuvette.
3. Add 15 µL of supernatant and obtain baseline oxygen production rate.
4. Add 15 µL of 1 M H_2O_2 to initiate the reaction and obtain oxygen production rate. (Final concentration of H_2O_2 = 9.8 mM).

Calculations:

Catalase activity in Units/mg heart wet weight.

Where 1 Unit = 1 µmol H_2O_2/min

Net O_2 production rate (Step 4 - Step 3) X 2 (as 2 hydrogen peroxides are consumed for each oxygen produced) divided by amount of tissue in 15 µL.

APPENDIX L- SUPEROXIDE DISMUTASE ASSAY

Sample Preparation:

1. Pipette 1 mL of tissue homogenate into centrifuge tube.
2. Centrifuge 1,500 x g for 10 minutes at 4°C.
3. Retain supernatant for analysis.

Solutions:

Solution A:

50 mM KH_2PO_4, 0.1 mM EDTA, 0.05 mM xanthine, 0.01 mM cyrochrome c, in dH_2O.

Potassium Cyanide (KCN):

2 mM in dH_2O.

Xanthine Oxidase (XO):

1:40 v:v in dH_2O.

Procedure:

1. Set up spectrophotometer to read the reaction for 3 minutes at a wavelength of 550 nm.
2. Determine baseline without supernatant. Baseline is the rate of superoxide/cyto c, reaction without SOD. To a 3 mL cuvette, add 1.4 mL of solution A. Place the cuvette in the spectrophotometer and start reaction with 10 µL of XO. Record the absorbance at 30 and 150 seconds after adding XO. Absorbance at 150 seconds – absorbance at 30 seconds = baseline absorbance change.

3. Determine rate with supernatant (containing MnSOD). To a cuvette, add 1.3 mL of solution A, 10 μL of supernatant, and 10 μL of KCN, which inhibits CuZnSOD (final concentration of KCN = 2.0 mM). Place the cuvette in the spectrophotometer and start reaction with 10 μL XO as in Step 2 and record absorbance at 30 and 150 seconds after adding xanthine oxidase. Absorbance at 150 seconds – absorbance at 30 seconds = sample absorbance change.

Calculations:

Percent inhibition of baseline = (baseline absorbance change – sample absorbance change)/baseline absorbance change.

Units MnSOD/mg heart wet weight = percent inhibition of baseline X 0.02 divided by mg heart tissue in cuvette. One unit = 50% inhibition of baseline.

APPENDIX M- RAW DATA

CF (mL/min/g heart wet weight)

sedentary (SED)

D	pre	5	10	15	20	25	30
119	11.8	13.6	15.3	14.1	11	10.8	11.1
120	14.3	14.3	15	14.1	11.8	11.8	12
124	12.8	15.6	15.3	11.6	11.4	11.7	11.9
125	14.7	13.1	13.7	13.3	11.3	11.8	12.2
126	12.4	14.5	14	14.2	10.8	10.6	10.2
127	12.9	13	13.7	9.6	9.5	9.6	9.6
131	15.6	14.1	14.6	16.8	16.6	14.4	15.2
mean	13.5	14.029	14.514	13.386	11.771	11.529	11.743
SD	1.3808	0.9013	0.7151	2.2682	2.25	1.5019	1.8091
n	7	7	7	7	7	7	7
SE	0.5219	0.3407	0.2703	0.8573	0.8504	0.5677	0.6838

2 days run (RUN)

D	pre	5	10	15	20	25	30
117	13.5	14.4	13.8	12.7	12.8	12.9	13.4
118	12.6	14.2	14.9	11.4	11.8	11.9	11.9
121	15.7	16.7	17	16.1	15.9	15.8	14.9
123	16.9	17.4	17.7	17.9	18.6	18.6	18.5
128	12.8	14.5	16	13.2	13.3	12.8	13.2
129	14.2	17.4	18.1	15.2	14.8	14.7	14.5
130	13.4	14.3	16.3	13.7	13.6	13.4	13.2
mean	14.157	15.557	16.257	14.314	14.4	14.3	14.229
SD	1.5904	1.5263	1.5263	2.2192	2.2811	2.2978	2.1211
n	7	7	7	7	7	7	7
SE	0.6011	0.5769	0.5769	0.8388	0.8622	0.8685	0.8017

sedentary/MPG (SED/MPG)

D	pre	5	10	15	20	25	30
133	18.8	16	16.8	19.7	19.7	19.9	21.4
134	13.1	14.5	15	12.4	10.7	10.7	11.1
135	13	17.7	16.8	13	12.6	12.4	12.4
136	13.1	16.1	13.5	11.3	11.3	11.3	11.3
137	16.4	21.4	15.9	14.9	15	14.6	15
138	14.4	17.5	16.1	12.4	11.8	11.3	11.7
145	14.8	15.8	16.2	14.6	14	14	14.4
147	15.7	15.5	15.1	13.6	13.7	13.9	14.7
182	21.2	17.6	17.1	21.3	21.8	21.4	21.9
183	14.9	16.3	16.6	13.5	13.4	13.2	13.5
194	15	15.3	16.4	13.1	13.2	13.6	13.2
195	14.9	12.1	13.1	12.6	11.3	11.6	12
mean	15.442	16.317	15.717	14.367	14.042	13.992	14.383
SD	2.4239	2.2156	1.2995	3.044	3.4068	3.3646	3.6369
n	12	12	12	12	12	12	12
SE	0.6997	0.6396	0.3751	0.8787	0.9835	0.9713	1.0499

2 days run/MPG (RUN/MPG)

D	pre	5	10	15	20	25	30
139	14.1	14.6	14.6	13.7	13.7	13.7	14.4
141	12.8	19.5	19.5	13.2	13.5	14.3	14
142	10.2	22	23.3	15.2	11.8	12.3	12.5
143	13.2	16.3	15.3	13.3	13.7	13.6	13.7
146	14.3	16.7	15.3	14.7	11.9	11.7	12
151	13.7	19.8	18.6	15.8	15.8	15.5	15.4
152	16.2	18.6	19.6	18.3	17.6	17.7	17.4
153	13	19.1	17.5	13.8	13.9	13.1	13.2
197	12.8	20.3	17.2	13.4	13.5	12.8	12.8
198	14.7	20.9	19.5	14.5	13.7	14	13.7
mean	13.5	18.78	18.04	14.59	13.91	13.87	13.91
SD	1.5628	2.2807	2.6357	1.5652	1.7045	1.7173	1.5673
n	10	10	10	10	10	10	10
SE	0.4942	0.7212	0.8335	0.495	0.539	0.543	0.4956

sedentary, L-NAME perfused (SED/L-N)

D	pre	5	10	15	20	25	30
157	12.2	13.6	12.2	10.4	9.8	9.9	9.7
158	11.8	12.9	11.6	10.1	10	8.2	8.4
159	10.6	13.4	10.7	9.2	8.3	8	6.9
160	11.8	16.3	11.2	10.8	10.5	10.4	8.8
171	10.7	11.5	13.5	8.6	8.4	8.7	9.2
172	8.5	16	12.6	9.5	9.1	9.2	9.2
mean	10.933	13.95	11.967	9.7667	9.35	9.0667	8.7
SD	1.356	1.8577	1.0132	0.8165	0.8961	0.9501	0.9839
n	6	6	6	6	6	6	6
SE	0.5536	0.7584	0.4137	0.3333	0.3658	0.3879	0.4017

2 days run, L-NAME perfused (RUN/L-N)

D	pre	5	10	15	20	25	30
161	10.9	16.8	12.3	10.7	10.8	10.7	10.6
162	11.7	15.5	13.2	11	11.3	10.2	11.2
163	10.9	14.4	14.2	10	10.3	10.1	10.6
165	13.5	16.6	14.6	13.4	13.7	14.1	14.3
166	12.8	15.8	13.6	11.4	11.2	11.3	10.9
168	11.3	16.2	12.5	11	11.1	10.7	10.6
mean	11.85	15.883	13.4	11.25	11.4	11.183	11.367
SD	1.0728	0.8727	0.9143	1.152	1.1832	1.4919	1.4569
n	6	6	6	6	6	6	6
SE	0.438	0.3563	0.3733	0.4703	0.483	0.6091	0.5948

2 days run/Cold, L-NAME perfused (CRUN/L-N)

D	pre	5	10	15	20	25	30
173	12.9	15	14.1	12.9	13.4	12.6	13.1
175	13.6	11.6	15.1	11.7	11.9	11.4	11.5
176	13	15.7	15.9	11.8	11.9	11.7	12
177	12.4	16	13	12.1	12.9	12.4	12.4
178	12.3	14.5	12.9	11.7	11.6	11.6	11.9
180	11.7	17	10.5	10.9	10.8	10.5	10.3
181	10.8	16.4	12.2	10.6	10.5	10.8	10.7
mean	12.386	15.171	13.386	11.671	11.857	11.571	11.7
SD	0.9227	1.7821	1.8206	0.7588	1.0406	0.7675	0.9644
n	7	7	7	7	7	7	7
SE	0.3488	0.6736	0.6881	0.2868	0.3933	0.2901	0.3645

LDH (mU/min/g heart wet weight)

sedentary (SED)

D	pre	5	10	15	20	25	30
119	8.3686	120.03	278.5	166.66	57.209		48.107
120	16.903	175.79	218.67	185.55	52.071		53.899
124	43.372	302.4	213.4	118.83	100.61		75.018
125	49.809	507.88	562.45	504.11	322.34		201.89
126	33.222	284.51	788.79	671.38	211.06		211.39
127	44.727	582.88	528.98	239.8	151.22		96.073
131	34.42	127.77	391.16	487.17	421.2		197.63
mean	32.974	300.18	425.99	339.07	187.96		126.29
SD	15.251	183.01	212.91	212.62	139.96		74.095
n	7	7	7	7	7		7
SE	5.7643	69.173	80.474	80.364	52.9		28.005

2 days run (RUN)

D	pre	5	10	15	20	25	30
117	9.5742	138.44	192.48	97.074	83.717		68.635
118	45.672	245.05	146.77	103.31	56.72		50.637
121	30.929	223.71	150.04	105.3	58.887		12.915
123	67.918	263.26	140.87	122.72	90.872		90.384
128	15.13	281.08	260.99	164.35	92.228		82.173
129	31.331	283.82	193.97	113.79	54.813		49.132
130	28.51	296.36	348.08	218.07	136.1		130.02
mean	32.723	247.39	204.74	132.09	81.906		69.128
SD	19.471	54.082	75.73	44.023	28.942		37.04
n	7	7	7	7	7		7
SE	7.3592	20.441	28.623	16.639	10.939		14

sedentary/MPG (SED/MPG)

D	pre	5	10	15	20	25	30
133	14.814	165.16	158.86	104.01	66.751		69.139
134	19.613	270.8	301.41	334.18	123.94		181.06
135	38.927	198.06	139	107.56	98.295		53.742
136	38.194	238.51	261.69	162.95	62.331		56.098
137	42.647	150.08	144.09	79.84	20.094		22.458
138	46.524	206.85	107.84	128	55.79		49.786
145	18.66	263.95	209.36	369.3	292.35		312.05
147	21.032	431.15	403.37	445.82	619.67		371.83
182	23.388	198.32	167.09	105.74	54.971		20.709
183	29.353	120.74	290.39	214.89	162.61		131.91
194	28.368	429.21	638.41	338.59	173.71		85.293
195	34.049	387.11	643.11	607.64	216.38		242.07
mean	29.631	255	288.72	249.88	162.24		133.01
SD	10.396	106.73	184.86	168	164.3		118.17
n	12	12	12	12	12		12
SE	3.0011	30.811	53.366	48.499	47.429		34.113

2 days run/MPG (RUN/MPG)

D	pre	5	10	15	20	25	30
139	18.888	144.96	161.07	133.87	109.04		41.985
141	20.173	161.34	142.9	88.414	46.807		23.167
142	21.702	173.36	220.32	318.6	91.124		74.86
143	9.3614	136.15	136.24	55.546	32.387		15.114
146	13.522	228.98	364.1	257.16	155.66		93.614
151	11.875	43.687	33.711	29.881	14.94		14.562
152	15.319	124.58	86.491	79.312	49.928		26.051
153	18.439	94.82	91.014	54.372	43.813		31.205
197	31.268	60.786	29.818	36.957	28.723		19.164
198	39.384	67.524	53.781	49.132	26.989		28.069
mean	19.993	123.62	131.95	110.32	59.941		36.779
SD	9.1432	57.662	101.45	99.256	44.625		26.642
n	10	10	10	10	10		10
SE	2.8913	18.234	32.081	31.387	14.112		8.4249

sedentary, L-NAME perfused (SED/L-N)

D	pre	5	10	15	20	25	30
157	12.498	161.82	177.85	99.981	40.929		47.39
158	13.948	240.92	301.65	225.23	151.3		92.007
159	20.882	336.84	349.91	314.63	202.1		197.37
160	11.158	147.71	103.26	60.424	55.436		40.913
171	14.334	63.434	179.78	115.21	84.726		55.097
172	12.726	172.73	122.12	67.374	32.986		28.273
mean	14.258	187.24	205.76	147.14	94.578		76.842
SD	3.4361	92.745	98.923	101.27	67.939		62.844
n	6	6	6	6	6		6
SE	1.4028	37.863	40.385	41.343	27.736		25.656

2 days run, L-NAME perfused (RUN/L-N)

D	pre	5	10	15	20	25	30
161	13.743	128.41	92.078	42.158	23.829		17.541
162	12.907	119.7	81.132	52.875	29.385		37.95
163	11.166	124.82	193.58	155.24	80.352		76.846
165	13.829	117.73	70.179	40.125	35.625		12.395
166	17.147	98.358	84.663	38.628	17.651		11.166
168	32.946	114.89	61.07	39.006	24.491		23.388
mean	16.956	117.32	97.117	61.338	35.222		29.881
SD	8.0715	10.493	48.51	46.305	22.911		24.985
n	6	6	6	6	6		6
SE	3.2952	4.2839	19.804	18.904	9.3536		10.2

2 days run/Cold, L-NAME perfused (CRUN/L-N)

D	pre	5	10	15	20	25	30
173	31.512	213.94	171.11	110.8	62.299		64.001
175	24.649	48.446	253.44	325.45	223.18		259.17
176	45.074	176.91	146.59	108.79	55.325		61.464
177	36.153	105.91	91.172	57.209	57.942		53.742
178	55.247	85.695	55.909	54.396	39.305		42.197
180	23.049	151.37	62.882	59.265	33.191		38.147
181	25.531	125.36	38.454	37.588	17.375		29.511
mean	34.459	129.66	117.08	107.64	69.802		78.319
SD	11.99	56.153	77.371	100.07	69.462		80.728
n	7	7	7	7	7		7
SE	4.5316	21.224	29.243	37.821	26.254		30.512

AF (mL/min/g heart wet weight)

sedentary (SED)

D	pre	15	20	25	30
119	38.9	0	10.6	12.9	18.4
120	40	0	14.2	19.4	20.2
124	45.1	29.9	34.8	31.6	33.7
125	51.1	0	10.3	10.7	11.3
126	28.9	0	8.6	10	11.7
127	40.7	24.3	24.2	23.8	21.3
131	44.7	0	0	15.9	17.7
mean	41.343	7.7429	14.671	17.757	19.186
SD	6.875	13.322	11.431	7.83	7.4968
n	7	7	7	7	7
SE	2.5985	5.0352	4.3204	2.9595	2.8335

2 days run (RUN)

D	pre	15	20	25	30
117	45.8	23.4	25.9	26.2	26.2
118	37.8	13.9	21	22.8	24.2
121	45.8	33.1	34.4	37.3	36.1
123	53.8	43.4	42	44	43.9
128	42.5	24.2	32.1	32.7	33.1
129	48.3	37.6	38.9	41.1	39.6
130	45.5	27.2	30	31.2	30.9
mean	45.643	28.971	32.043	33.614	33.429
SD	4.9169	9.8639	7.2523	7.7036	7.0568
n	7	7	7	7	7
SE	1.8584	3.7282	2.7411	2.9117	2.6672

sedentary/MPG (SED/MPG)

D	pre	15	20	25	30
133	42.4	16.5	17.5	17.9	25
134	39.9	0	11	13	14.2
135	42.7	34.2	35.9	37.1	38
136	40.6	27.1	29.4	29.7	29.7
137	55.7	41.3	44	44.5	46.8
138	44.5	21.7	22.5	22.5	28.9
145	52.3	0	0	0	0
147	46.9	0	0	0	0
182	51.3	33.7	34.3	34.9	34.3
183	52	22.8	25.2	26.3	26.1
194	54	24.9	26.6	27.1	27.4
195	53.3	0	12	12.9	13.4
mean	47.967	18.517	21.533	22.158	23.65
SD	5.7369	15.118	13.888	13.99	14.316
n	12	12	12	12	12
SE	1.6561	4.3641	4.009	4.0387	4.1326

2 days run/MPG (RUN/MPG)

D	pre	15	20	25	30
139	43.5	27.5	29.9	30.3	30.9
141	41.8	23.7	26.1	26.9	27.2
142	35.2	0	6.2	9.3	21.9
143	39.7	29.7	31.8	32.1	32.4
146	45.1	0	12.9	15.7	17.3
151	44.1	39.1	39.7	38.5	36.7
152	42.7	33	34.8	35.8	36.1
153	40.6	31.5	31.8	31.7	30.7
197	44.7	40.2	38.8	38.6	39.1
198	49.7	44.9	45.1	44.2	42.6
mean	42.71	26.96	29.71	30.31	31.49
SD	3.8272	15.541	12.029	10.701	7.7694
n	10	10	10	10	10
SE	1.2103	4.9145	3.8039	3.3838	2.4569

sedentary, L-NAME perfused (SED/L-N)

D	pre	15	20	25	30
157	42.6	20.6	20.8	22.3	23.7
158	47.3	0	0	5.4	7
159	39.4	0	0	0	3.2
160	50.9	38.8	38.3	36.8	21.2
171	46.4	16.7	18.5	27.2	27.1
172	38.1	28.9	30.3	31.7	32.3
mean	44.117	17.5	17.983	20.567	19.083
SD	4.9418	15.533	15.617	14.748	11.515
n	6	6	6	6	6
SE	2.0175	6.3414	6.3757	6.0208	4.7012

2 days run, L-NAME perfused (RUN/L-N)

D	pre	15	20	25	30
161	47.7	37.3	37.5	37.7	38.6
162	53.9	40.2	36.3	22.4	39.9
163	45.6	21	27.3	29.3	29.6
165	51.2	41	42.6	43.2	42.3
166	49	37.4	39	36.9	37
168	47.6	37.7	38.5	38.5	38.6
mean	49.167	35.767	36.867	34.667	37.667
SD	2.9629	7.4007	5.1446	7.4963	4.3293
n	6	6	6	6	6
SE	1.2096	3.0213	2.1003	3.0604	1.7674

2 days run/Cold, L-NAME perfused (CRUN/L-N)

D	pre	15	20	25	30
173	45.1	33.2	35	32.4	32.1
175	48.9	16.9	29.1	30.7	31.6
176	49.2	34.1	37	36.8	37.8
177	49.8	40.4	41.7	41.3	41
178	46.6	38.6	38.8	39	37.8
180	41.3	30.1	40.1	40.8	41.2
181	44.1	40.3	41.1	40.1	40.8
mean	46.429	33.371	37.543	37.3	37.471
SD	3.1271	8.2393	4.3988	4.219	4.1023
n	7	7	7	7	7
SE	1.182	3.1142	1.6626	1.5946	1.5505

CO (mL/min/g heart wet weight)

sedentary (SED)

D	pre	15	20	25	30
119	50.7	14.1	21.6	23.7	29.5
120	54.3	14.1	26	31.2	32.2
124	57.9	41.5	46.2	43.3	45.6
125	65.8	13.3	21.6	22.5	23.5
126	41.3	14.2	19.4	20.6	21.9
127	53.6	33.9	33.7	33.4	30.9
131	60.3	16.8	16.6	30.3	32.9
mean	54.843	21.129	26.443	29.286	30.929
SD	7.7631	11.583	10.3	7.8603	7.7405
n	7	7	7	7	7
SE	2.9342	4.3778	3.8932	2.9709	2.9256

2 days run (RUN)

D	pre	15	20	25	30
117	59.3	36.1	38.7	39.1	39.6
118	50.4	25.3	32.8	34.7	36.1
121	61.5	49.2	50.3	53.1	51
123	70.7	61.3	60.6	62.6	62.4
128	55.3	37.4	45.4	45.5	46.3
129	62.5	52.8	53.7	55.8	54.1
130	58.8	40.9	43.6	44.6	44.1
mean	59.786	43.286	46.443	47.914	47.657
SD	6.3094	12.01	9.3382	9.7798	8.9723
n	7	7	7	7	7
SE	2.3847	4.5394	3.5295	3.6964	3.3912

sedentary/MPG (SED/MPG)

D	pre	15	20	25	30
133	61.2	36.2	37.2	37.8	46.4
134	53	12.4	21.7	23.7	25.3
135	55.7	47.2	48.5	49.5	50.4
136	53.7	38.4	40.7	41	41
137	72.1	56.2	59	59.1	61.8
138	58.9	34.1	34.3	33.8	40.6
145	67.1	14.6	14	14	14.4
147	62.6	13.6	13.6	13.9	14.7
182	72.5	55	56.5	56.3	56.2
183	66.9	36.3	38.6	39.5	39.6
194	69	38	39.8	40.2	40.6
195	68.2	12.6	23.3	24.5	25.4
mean	63.408	32.883	35.6	36.108	38.033
SD	6.9158	16.112	15.084	14.914	15.276
n	12	12	12	12	12
SE	1.9964	4.6512	4.3543	4.3053	4.4098

2 days run/MPG (RUN/MPG)

D	pre	15	20	25	30
139	57.6	41.2	43.6	44	45.3
141	54.6	36.9	39.6	41.2	41.2
142	45.2	15.2	18	21.6	34.4
143	52.9	43	45.5	45.7	46.1
146	59.4	14.7	24.8	27.4	29.3
151	57.8	54.9	55.5	54	52.1
152	58.9	51.3	52.4	53.5	53.5
153	53.6	45.3	45.7	44.8	43.9
197	57.5	53.6	52.3	51.4	51.9
198	64.4	59.4	58.8	58.2	56.3
mean	56.19	41.55	43.62	44.18	45.4
SD	5.0792	15.6	13.16	11.714	8.642
n	10	10	10	10	10
SE	1.6062	4.9332	4.1615	3.7044	2.7328

sedentary, L-NAME perfused (SED/L-N)

D	pre	15	20	25	30
157	54.8	31	30.6	32.2	33.4
158	59.1	10.1	10	13.6	15.4
159	50	9.2	8.3	8	10.1
160	62.7	49.6	48.8	47.2	30
171	57.1	25.3	26.9	35.9	36.3
172	46.6	38.4	39.4	40.9	41.5
mean	55.05	27.267	27.333	29.633	27.783
SD	5.9346	15.885	16	15.534	12.355
n	6	6	6	6	6
SE	2.4228	6.4849	6.532	6.3415	5.0438

2 days run, L-NAME perfused (RUN/L-N)

D	pre	15	20	25	30
161	58.6	48	48.3	48.4	49.2
162	65.6	51.2	47.6	32.6	51.1
163	56.5	31	37.6	39.4	40.2
165	64.7	54.4	56.3	57.3	56.6
166	61.8	48.8	50.2	48.2	47.9
168	58.9	48.7	49.6	49.2	49.2
mean	61.017	47.017	48.267	45.85	49.033
SD	3.6307	8.1918	6.0708	8.6199	5.3084
n	6	6	6	6	6
SE	1.4822	3.3443	2.4784	3.5191	2.1671

2 days run/Cold, L-NAME perfused (CRUN/L-N)

D	pre	15	20	25	30
173	58	46.1	48.4	45	45.2
175	62.5	28.6	41	42.1	43.1
176	62.2	45.9	48.9	48.5	49.8
177	62.2	52.5	54.6	53.7	53.4
178	58.9	50.3	50.4	50.6	49.7
180	53	41	50.9	51.3	51.5
181	54.9	50.9	51.6	50.9	51.5
mean	58.814	45.043	49.4	48.871	49.171
SD	3.7927	8.2272	4.2218	4.0269	3.6976
n	7	7	7	7	7
SE	1.4335	3.1096	1.5957	1.522	1.3976

HR (beats/min)

sedentary (SED)

D	pre	15	20	25	30
119	237	98	243	237	237
120	279	271	304	303	298
124	293	294	295	294	295
125	295			244	244
126	254	195	250	256	209
127	296	295	295	295	294
131	283	218	242	257	258
mean	276.71	228.5	271.5	269.43	262.14
SD	22.765	75.87	29.345	27.135	34.6
n	7	6	6	7	7
SE	8.6043	30.974	11.98	10.256	13.077

2 days run (RUN)

D	pre	15	20	25	30
117	302	299	297	288	293
118	329	299	307	313	319
121	294	294	294	293	294
123	293	293	291	293	293
128	294	304	294	294	295
129	294	296	295	295	294
130	294	295	295	296	295
mean	300	297.14	296.14	296	297.57
SD	13.153	3.8048	5.113	7.9162	9.4843
n	7	7	7	7	7
SE	4.9713	1.4381	1.9325	2.9921	3.5847

sedentary/MPG (SED/MPG)

D	pre	15	20	25	30
133	295	295	295		295
134	261		240	241	247
135	295	295	294	295	295
136	295	294	295	295	295
137	295	295	294	295	295
138	294	295	295	295	295
145	298	163	208	115	219
147	270	220	156	100	150
182	295	295	295	295	295
183	279	257	265	269	272
194	295	295	295	295	295
195	323		237	235	235
mean	291.25	270.4	264.08	248.18	265.67
SD	15.557	45.268	45.438	73.148	45.636
n	12	10	12	11	12
SE	4.4909	14.315	13.117	22.055	13.174

2 days run/MPG (RUN/MPG)

D	pre	15	20	25	30
139	295	295	295	295	295
141	269	230	237	240	246
142	254	103	174	170	285
143	295	295	295	295	295
146	281	229	248	250	253
151	295	295	295	295	295
152	295	295	295	295	295
153	295	295	295	295	295
197	295	295	295	295	295
198	295	295	295	295	295
mean	286.9	262.7	272.4	272.5	284.9
SD	14.518	62.372	40.969	41.65	18.988
n	10	10	10	10	10
SE	4.5909	19.724	12.956	13.171	6.0045

sedentary, L-NAME perfused (SED/L-N)

D	pre	15	20	25	30
157	267	228	216	226	241
158	277	235	228	256	258
159	270	204	180	181	210
160	295	293	294	292	194
171	295	295	295	295	295
172	301	283	278	289	288
mean	284.17	256.33	248.5	256.5	247.67
SD	14.593	38.852	47.479	45.672	40.786
n	6	6	6	6	6
SE	5.9577	15.861	19.383	18.645	16.651

2 days run, L-NAME perfused (RUN/L-N)

D	pre	15	20	25	30
161	295	295	295	295	295
162	295	295	295	295	295
163	289	331	320	323	304
165	295	295	295	295	295
166	294	295	294	294	295
168	319	294	294	295	294
mean	297.83	300.83	298.83	299.5	296.33
SD	10.629	14.784	10.381	11.52	3.7771
n	6	6	6	6	6
SE	4.3391	6.0355	4.2381	4.7028	1.542

2 days run/Cold, L-NAME perfused (CRUN/L-N)

D	pre	15	20	25	30
173	295	295	295	295	295
175	294		293	293	293
176	295	295	295	295	295
177	295	295	295	295	295
178	295	295	295	295	295
180	295	295	295	295	295
181	295	295	295	295	295
mean	294.86	295	294.71	294.71	294.71
SD	0.378	0	0.7559	0.7559	0.7559
n	7	6	7	7	7
SE	0.1429	0	0.2857	0.2857	0.2857

SP (mmHg)

sedentary (SED)

D	pre	15	20	25	30
119	126	111	97	99	107
120	119	111	107	111	114
124	113	102	103	101	102
125	108		98	93	95
126	111	106	90	90	101
127	110	95	95	95	95
131	112	92	106	92	95
mean	114.14	102.83	99.429	97.286	101.29
SD	6.2564	8.0353	6.1875	7.1813	7.2276
n	7	6	7	7	7
SE	2.3647	3.2804	2.3387	2.7143	2.7318

2 days run (RUN)

D	pre	15	20	25	30
117	106	104	107	110	111
118	113	99	103	106	109
121	113	105	107	107	109
123	107	104	103	106	107
128	116	120	109	113	115
129	113	107	108	109	110
130	109	100	101	103	103
mean	111	105.57	105.43	107.71	109.14
SD	3.6968	6.9488	3.0472	3.2514	3.671
n	7	7	7	7	7
SE	1.3973	2.6264	1.1518	1.2289	1.3875

sedentary/MPG (SED/MPG)

D	pre	15	20	25	30
133	108	98	94		99
134	121		97	98	99
135	102	94	95	97	97
136	106	91	91	91	91
137	112	110	111	111	111
138	108	92	93	93	95
145	110	85	87	97	97
147	113	86	88	90	97
182	112	103	103	104	104
183	111	104	105	106	106
194	122	96	97	98	100
195	105		96	96	99
mean	110.83	95.9	96.417	98.273	99.583
SD	5.9365	8.0201	6.9995	6.4201	5.2822
n	12	10	12	11	12
SE	1.7137	2.5362	2.0206	1.9357	1.5248

2 days run/MPG (RUN/MPG)

D	pre	15	20	25	30
139	109	96	100	101	101
141	106	107	107	109	108
142	113	106	101	105	94
143	102	99	100	101	100
146	115	96	98	100	101
151	112	105	106	106	106
152	108	102	102	103	104
153	102	95	96	97	96
197	114	111	112	114	114
198	107	103	103	103	104
mean	108.8	102	102.5	103.9	102.8
SD	4.6857	5.3955	4.7199	4.8865	5.808
n	10	10	10	10	10
SE	1.4817	1.7062	1.4926	1.5452	1.8367

sedentary, L-NAME perfused (SED/L-N)

D	pre	15	20	25	30
157	112	102	103	104	102
158	107	83	93	82	84
159	116	85	90	99	87
160	115	104	103	103	113
171	108	85	84	89	89
172	93	93	94	96	97
mean	108.5	92	94.5	95.5	95.333
SD	8.4083	9.2087	7.4498	8.5499	10.93
n	6	6	6	6	6
SE	3.4327	3.7594	3.0414	3.4905	4.4622

2 days run, L-NAME perfused (RUN/L-N)

D	pre	15	20	25	30
161	108	102	102	102	103
162	110	103	107	118	106
163	111	94	99	103	104
165	111	108	106	108	109
166	116	111	112	113	113
168	111	104	104	105	106
mean	111.17	103.67	105	108.17	106.83
SD	2.6394	5.8195	4.4721	6.2423	3.656
n	6	6	6	6	6
SE	1.0775	2.3758	1.8257	2.5484	1.4926

2 days run/Cold, L-NAME perfused (CRUN/L-N)

D	pre	15	20	25	30
173	122	116	116	118	119
175	120		114	118	120
176	110	107	107	106	108
177	114	111	111	112	114
178	110	106	106	110	110
180	114	104	105	109	113
181	111	107	108	110	112
mean	114.43	108.5	109.57	111.86	113.71
SD	4.8255	4.3243	4.1975	4.5617	4.424
n	7	6	7	7	7
SE	1.8239	1.7654	1.5865	1.7242	1.6721

COxSP (mmHgxmL/min/g heart wet weight)

sedentary (SED)

D	pre	15	20	25	30
119	6388	1565	2095	2346	3157
120	6462	1565	2782	3463	3671
124	6543	4233	4759	4373	4651
125	7106		2117	2093	2233
126	4584	1505	1746	1854	2212
127	5896	3221	3202	3173	2936
131	6754	1546	1760	2788	3126
mean	6247.6	2272.5	2637.3	2870	3140.9
SD	820.31	1171.4	1077.8	877.47	845.68
n	7	6	7	7	7
SE	310.05	478.23	407.38	331.65	319.64

2 days run (RUN)

D	pre	15	20	25	30
117	6286	3754	4141	4301	4396
118	5695	2508	3378	3678	3935
121	6950	5166	5382	5682	5559
123	7565	6375	6242	6636	6677
128	6415	4488	4949	5142	5325
129	7063	5650	5800	6082	5951
130	6409	4090	4404	4594	4542
mean	6626.1	4575.9	4899.4	5159.3	5197.9
SD	612.6	1286.7	998.69	1045.3	963.47
n	7	7	7	7	7
SE	231.54	486.34	377.47	395.09	364.16

sedentary/MPG (SED/MPG)

D	pre	15	20	25	30
133	6610	3548	3797		4594
134	6413		2105	2323	2505
135	5681	4437	4608	4802	4889
136	5692	3494	3704	3731	3731
137	8075	6182	6549	6560	6860
138	6361	3137	3190	3143	3857
145	7381	1241	1218	1358	1397
147	7074	1170	1197	1251	1426
182	8120	5665	5820	5855	5845
183	7426	3775	4053	4187	4198
194	8418	3648	3861	3940	4050
195	7161		2237	2352	2515
mean	7034.3	3629.7	3528.3	3591.1	3822.3
SD	910.56	1610.9	1668.2	1723.6	1660.3
n	12	10	12	11	12
SE	262.86	509.4	481.57	519.7	479.29

2 days run/MPG (RUN/MPG)

D	pre	15	20	25	30
139	6278	3955	4360	4444	4575
141	5788	3948	4237	4491	4450
142	5130	1611	1818	2268	3234
143	5396	4257	4550	4616	4610
146	6831	1411	2430	2740	2959
151	6474	5765	5883	5724	5523
152	6361	5233	5345	5511	5564
153	5467	4304	4387	4346	4214
197	6555	5950	5858	5860	5917
198	6891	6118	6056	5995	5855
mean	6117.1	4255.2	4492.4	4599.5	4690.1
SD	627.02	1661.5	1432.7	1274.8	1040.3
n	10	10	10	10	10
SE	198.28	525.42	453.06	403.12	328.98

sedentary, L-NAME perfused (SED/L-N)

D	pre	15	20	25	30
157	6138	3162	3152	3349	3407
158	6324	838	930	1115	1294
159	5800	782	747	792	879
160	7211	5158	5026	4862	3390
171	6167	2151	2260	3195	3231
172	4334	3571	3704	3926	4026
mean	5995.7	2610.3	2636.5	2873.2	2704.5
SD	941.69	1697.8	1657.7	1601	1289.1
n	6	6	6	6	6
SE	384.44	693.13	676.74	653.61	526.27

2 days run, L-NAME perfused (RUN/L-N)

D	pre	15	20	25	30
161	6329	4896	4927	4937	5068
162	7216	5274	5093	3847	5417
163	6272	2914	3722	4058	4181
165	7182	5875	5968	6188	6169
166	7169	5417	5622	5447	5413
168	6538	5065	5158	5166	5215
mean	6784.3	4906.8	5081.7	4940.5	5243.8
SD	452.31	1032.4	768.91	876.15	644.34
n	6	6	6	6	6
SE	184.66	421.46	313.91	357.69	263.05

2 days run/Cold, L-NAME perfused (CRUN/L-N)

D	pre	15	20	25	30
173	7076	5348	5614	5310	5379
175	7500		4674	4968	5172
176	6842	4911	5232	5141	5378
177	7091	5828	6061	6014	6088
178	6479	5332	5342	5566	5467
180	6042	4264	5345	5592	5820
181	6094	5446	5573	5599	5768
mean	6732	5188.2	5405.9	5455.7	5581.7
SD	547.16	539.07	423.1	346.67	319.22
n	7	6	7	7	7
SE	206.81	220.08	159.92	131.03	120.66

Efficiency (COxSP/O2)

sedentary (SED)

D	pre	15	20	25	30
119	767.88	183.76	285.97	326.16	427.05
120	707.18	195.07	351.88	431.58	451.87
124	731.29	522.05	588.8	525.68	549.71
125	664.01		261.29	272.04	282.46
126	619.23	192.35	252.21	275.88	343.14
127	634.8	443.81	454.86	445.45	414.41
131	778.68	216.02	228.99	400.02	426.68
mean	700.44	292.18	346.29	382.4	413.61
SD	63.113	150.19	131.72	94.8	84.167
n	7	6	7	7	7
SE	23.854	61.315	49.786	35.831	31.812

2 days run (RUN)

D	pre	15	20	25	30
117	692.9	466.23	482.86	502.12	495.56
118	626.02	322.58	406.06	439.03	473.06
121	789.08	590.92	617.68	636.5	646.6
123	721.99	789.68	793.36		750.35
128	835.29	505.95	579.6	631.63	632.3
129	749.09	580.8	607.58	635.55	625.63
130	720.31	465.74	498.19	527.44	526.94
mean	733.52	531.7	569.33	562.04	592.92
SD	67.33	144.63	124.69	84.51	98.729
n	7	7	7	6	7
SE	25.449	54.664	47.13	34.501	37.316

sedentary/MPG (SED/MPG)

D	pre	15	20	25	30
133	727.94	549.09	566.89		828.85
134	756.63		299.89	338.69	351.52
135	707.12	560.44	581.42	601.33	603.79
136	596.85	426.49	437.05	440.82	439.65
137	709.48	610.15	622.82	631.06	637.84
138	631.05	377.59	391.8	391.75	464.96
145	748.82	167.98	167.63	173.21	177.03
147	729.08	178.11	176.51	185.19	205.96
182	667.28	525.62	498.08	529.2	516.24
183	724.4	423.68	448.76	474.14	451.33
194	897.92	459.53	480.3	472.6	502.16
195	733.75		316.74	340.2	362.6
mean	719.19	427.87	415.66	416.2	461.83
SD	74.045	152.21	150.23	150.05	180.81
n	12	10	12	11	12
SE	21.375	48.134	43.367	45.241	52.197

2 days run/MPG (RUN/MPG)

D	pre	15	20	25	30
139					
141					
142	715.42	193.05	275.61	338.95	426.93
143	647.84	522.15	548.05	554.59	564.59
146	775.47	199.56	325.68	373.51	388.32
151	713.83	597.17	588.22	577.92	556.89
152	579.14	471.1	466.5	501.38	513.27
153	548.29	437.43	424.21	440.58	418.4
197	807.74	676.87	652.52	683.3	679.8
198	734.76	663.41	661.74	624.22	614.04
mean	690.31	470.09	492.82	511.81	520.28
SD	91.445	188.98	144.77	120.97	102.89
n	8	8	8	8	8
SE	32.331	66.815	51.183	42.769	36.375

sedentary, L-NAME perfused (SED/L-N)

D	pre	15	20	25	30
157	676.23	410.86	423.76	445.11	461.55
158	657.59	128.04	129.53	164.62	189.95
159	733.47	147.57	135.95	143.69	165.44
160	773.55	606.85	603.61	592.52	499
171	763.38	343.57	361.62	491.62	467.64
172	678.04	524.99	566.11	586.18	593.77
mean	713.71	360.31	370.1	403.96	396.22
SD	49.53	194.91	204.26	201.58	175.93
n	6	6	6	6	6
SE	20.22	79.571	83.387	82.293	71.825

2 days run, L-NAME perfused (RUN/L-N)

D	pre	15	20	25	30
161	755.06	598.91	596.34	605.51	624.98
162	821.24	628.38	586.86	488.55	638.92
163	787.16	406.98	483.1	535.71	527.32
165	764.37	679.74	669.16	684.66	701.46
166	729.27	641.26	670.18	641	669.28
168	782.93	627.32	624.58	652.44	665.74
mean	773.34	597.1	605.04	601.31	637.95
SD	31.414	96.767	69.298	75.065	60.326
n	6	6	6	6	6
SE	12.825	39.505	28.291	30.645	24.628

2 days run/Cold, L-NAME perfused (CRUN/L-N)

D	pre	15	20	25	30
173	765.03	639.77	643.56	650.35	641.58
175	705.21		530.06	577.2	604.49
176	677.36	554.18	577.74	578.16	588.92
177	739.79	643.06	615.79	634.82	646.01
178	709.9	612.54	612.39	636.38	605.29
180	662.06	509.37	645.25	686.3	735.74
181	734.71	667.24	680.46	669.8	698.27
mean	713.44	604.36	615.04	633.29	645.76
SD	36.125	60.522	49.374	42.094	53.92
n	7	6	7	7	7
SE	13.654	24.708	18.662	15.91	20.38

HSP70 (% of standard)

(SED)

D	
119	11.3
120	82.5
124	83.4
125	57.5
126	83.7
127	30.2
131	49.6
mean	56.8857
SD	28.6407
n	7
SE	10.8252

(RUN)

D	
117	105.3
118	116.3
121	121.8
123	168.4
128	166.6
129	159.5
130	165.3
mean	143.314
SD	27.5517
n	7
SE	10.4136

(SED/MPG)

D	
133	44.4
134	64
135	77.1
136	33
137	69.5
138	63.5
182	86.8
183	83.2
mean	65.1875
SD	18.6106
n	8
SE	6.57982

(RUN/MPG)

D	
139	172.5
141	192.4
142	170.7
143	185
146	47.6
151	126.7
152	138.6
153	118.1
mean	143.95
SD	47.5708
n	8
SE	16.8188

(SED/L-N)

D	
157	28
158	99.3
159	77.6
160	32.7
171	30.1
172	35.4
mean	50.5167
SD	30.2758
n	6
SE	12.36

(RUN/L-N)

D	
161	143.2
162	95.4
163	115.7
165	165.5
166	113.1
168	133.2
mean	127.683
SD	24.8989
n	6
SE	10.165

(CRUN/L-N)

D	
173	53.7
175	7.5
176	51.7
177	47.8
178	47.3
180	62.7
181	77.9
mean	49.8
SD	21.5034
n	7
SE	8.12753

iNOS (% of standard)

(SED)	
D	
119	564.9
120	201.5
124	165.7
125	178.8
126	237.6
127	260
131	236.2
mean	263.529
SD	137.145
n	7
SE	51.8361

(RUN)	
D	
117	108.7
118	639
121	137.3
123	485.6
128	349.8
129	259.1
130	185.3
mean	309.257
SD	195.425
n	7
SE	73.8635

(SED/MPG)	
D	
133	109.2
134	174.1
135	303.5
136	180.5
137	132.2
138	115.3
182	801.7
183	221
mean	254.688
SD	230.011
n	8
SE	81.3213

(RUN/MPG)	
D	
139	271.8
141	107.6
142	119.3
143	725
146	166.5
151	124.5
152	218.8
153	149.5
mean	235.375
SD	205.477
n	8
SE	72.6471

(SED/L-N)	
D	
157	391.2
158	287.6
159	339.2
160	279.3
171	414.8
172	312.2
mean	337.383
SD	55.4601
n	6
SE	22.6415

(RUN/L-N)	
D	
161	105.8
162	28.3
163	135.4
165	360.7
166	418.8
168	378.9
mean	237.983
SD	167.077
n	6
SE	68.2091

(CRUN/L-N)	
D	
173	191.5
175	72.5
176	191.6
177	193.6
178	155
180	270.5
181	589.9
mean	237.8
SD	166.06
n	7
SE	62.7648

eNOS (% of standard)

(SED)	
D	
119	50
120	48.2
124	46.8
125	21.8
126	22.4
127	57.6
131	43.6
mean	41.4857
SD	13.9149
n	7
SE	5.25934

(RUN)	
D	
117	51
118	75.9
121	67.7
123	64.9
128	37.9
129	50.4
130	20.7
mean	52.6429
SD	18.9881
n	7
SE	7.17681

(SED/MPG)	
D	
133	9.7
134	10.8
135	39.4
136	37.6
137	40.2
138	32.6
182	62.8
183	49
mean	35.2625
SD	17.9401
n	8
SE	6.3428

(RUN/MPG)	
D	
139	50.5
141	45.8
142	38.6
143	75.1
146	73.1
151	81.3
152	39.2
153	13.7
mean	52.1625
SD	22.9536
n	8
SE	8.11533

(SED/L-N)	
D	
157	52.9
158	40.1
159	47.1
160	72.3
171	75.7
172	94
mean	63.6833
SD	20.4216
n	6
SE	8.33708

(RUN/L-N)	
D	
161	31.6
162	32.9
163	26.2
165	19.9
166	48.8
168	74.5
mean	38.9833
SD	19.8842
n	6
SE	8.11769

(CRUN/L-N)	
D	
173	84.5
175	59.1
176	68
177	32.8
178	114.3
180	112.8
181	113.1
mean	83.5143
SD	31.8601
n	7
SE	12.042

CAT (U/mg heart wet weight)

(SED)

D	
119	1.6133
120	1.3067
124	1.96
125	1.42
126	1.36
127	1.6933
131	1.3333
mean	1.52666
SD	0.24083
n	7
SE	0.09102

(RUN)

D	
117	1.7467
118	1.4933
121	1.1733
123	1.6932
128	1.7333
129	1.5933
130	2.1867
mean	1.65997
SD	0.30565
n	7
SE	0.11552

(SED/MPG)

D	
133	1.8267
134	3.2667
135	2.9733
136	2.52
137	2.8267
138	3.2133
182	2.1467
183	1.8667
mean	2.58001
SD	0.58012
n	8
SE	0.2051

(RUN/MPG)

D	
139	3.4933
141	3.46
142	3.4667
143	3.2267
146	1.5
151	2.3467
152	2.1867
153	2.4933
mean	2.77168
SD	0.74662
n	8
SE	0.26397

(SED/L-N)

D	
157	1.6667
158	1.6267
159	1.56
160	1.6667
171	1.84
172	1.7333
mean	1.68223
SD	0.09592
n	6
SE	0.03916

(RUN/L-N)

D	
161	1.72
162	1.5333
163	1.6533
165	2.4667
166	1.8933
168	1.9467
mean	1.86888
SD	0.33024
n	6
SE	0.13482

(CRUN/L-N)

D	
173	1.8533
175	1.92
176	2.0533
177	1.72
178	1.72
180	1.6667
181	1.8133
mean	1.82094
SD	0.13483
n	7
SE	0.05096

MnSOD (U/mg heart wet weight)

(SED)	
D	
119	1.336
120	1.7509
124	1.9053
125	2.0733
126	2.2293
127	1.5547
131	1.6347
mean	1.78346
SD	0.30913
n	7
SE	0.11684

(RUN)	
D	
117	1.7467
118	1.7786
121	1.4912
123	1.5653
128	1.896
129	1.4707
130	1.7453
mean	1.67054
SD	0.1618
n	7
SE	0.06115

(SED/MPG)	
D	
133	
134	1.6067
135	1.8827
136	1.544
137	1.8933
138	1.852
182	
183	
mean	1.75574
SD	0.16685
n	5
SE	0.07462

(RUN/MPG)	
D	
139	2.052
141	2.0427
142	1.6213
143	1.6907
146	1.5573
151	1.7533
152	1.9693
153	1.9373
mean	1.82799
SD	0.19589
n	8
SE	0.06926

(CRUN/L-N)	
D	
173	
175	
176	0.952
177	1.03
178	0.693
180	0.982
181	0.834
mean	0.8982
SD	0.13557
n	5
SE	0.06063

Mass

(SED)

D	body (g)	heart (mg)	ratio (g/mg)
119	310	840	0.369
120	330	920	0.3587
124	360	1040	0.3462
125	330	870	0.3793
126	362	960	0.3771
127	304	880	0.3455
131	360	940	0.383
mean	336.57	921.43	0.3655
SD	24.487	66.94	0.0156
n	7	7	7
SE	9.2553	25.301	0.0059

(RUN)

D	body (g)	heart (mg)	ratio (g/mg)
117	290	910	0.3187
118	336	1000	0.336
121	350	940	0.3723
123	306	850	0.36
128	330	1020	0.3235
129	362	1040	0.3481
130	322	940	0.3426
mean	328	957.14	0.343
SD	24.739	67.011	0.0191
n	7	7	7
SE	9.3503	25.328	0.0072

sedentary/MPG (SED/MPG)

D	body (g)	heart (mg)	ratio (g/mg)
133	338	960	0.3521
134	336	950	0.3537
135	354	940	0.3766
136	362	930	0.3892
137	372	890	0.418
145	320	860	0.3721
147	336	880	0.3818
138	366	940	0.3894
182	350	950	0.3684
183	344	880	0.3909
194	358	970	0.3691
195	324	860	0.3767
mean	346.67	917.5	0.3782
SD	16.456	40.48	0.0179
n	12	12	12
SE	4.7503	11.686	0.0052

2 days run/MPG (RUN/MPG)

D	body (g)	heart (mg)	ratio (g/mg)
139	366	1050	0.3486
141	330	930	0.3548
142	326	930	0.3505
143	336	1040	0.3231
146	352	920	0.3826
151	342	1010	0.3386
152	352	960	0.3667
153	340	940	0.3617
197	350	1010	0.3465
198	318	930	0.3419
mean	341.2	972	0.3515
SD	14.336	50.288	0.0164
n	10	10	10
SE	4.5333	15.902	0.0052

(SED/L-N)

D	body (g)	heart (mg)	ratio (g/mg)
157	350	910	0.3846
158	354	910	0.389
159	358	940	0.3809
160	368	1000	0.368
171	324	920	0.3522
172	316	920	0.3435
mean	345	933.33	0.3697
SD	20.425	34.448	0.0185
n	6	6	6
SE	8.3387	14.063	0.0076

(RUN/L-N)

D	body (g)	heart (mg)	ratio (g/mg)
161	312	920	0.3391
162	300	870	0.3448
163	312	920	0.3391
165	312	940	0.3319
166	312	900	0.3467
168	292	880	0.3318
mean	306.67	905	0.3389
SD	8.641	26.646	0.0062
n	6	6	6
SE	3.5277	10.878	0.0025

(CRUN/L-N)

D	body (g)	heart (mg)	ratio (g/mg)
173	336	1080	0.3111
175	294	920	0.3196
176	292	880	0.3318
177	312	950	0.3284
178	350	1100	0.3182
180	362	1070	0.3383
181	316	930	0.3398
mean	323.14	990	0.3267
SD	27.052	90.185	0.0108
n	7	7	7
SE	10.225	34.087	0.0041

Body Temperature (°C)

Sedentary		Run			Cold Run		
D	0 min	D	30 min	60 min	D	30 min	60 min
173	37.6	107	40.8	41.7	173	37.3	34.1
174	37.2	108	40.7	41	174	35.4	32.3
175	37.8	109	40.8	41.7	175	37.3	35.9
176	37.7	110	40.4	40.4	176	36.8	36
177	38	111	40.4	41.3	177	35.3	33.7
178	38				178	35.2	33.8
mean	37.717		40.62	41.22		36.217	34.3
SD	0.2994		0.2049	0.545		1.0226	1.4213
n	6		5	5		6	6
SE	0.1222		0.0917	0.2437		0.4175	0.5802

Glossary

Anti-Oxidant - A substance that can reduce or destroy oxidants.

Aortic Flow (AF) - The volume of blood flowing through the aorta per minute, normalized for the size of the heart in milliliters per minute per gram of heart wet weight.

Cardiac Function (COxSP) - An index of external work that an isolated working heart is performing during perfusion. Calculated as the product of the cardiac output and peak systolic pressure.

Cardiac Output (CO) - The volume of blood the left ventricle of the heart ejects per minute, normalized for the size of the heart. Calculated as the sum of the coronary flow and the aortic flow in milliliters per minute per gram of heart wet weight.

Cardioprotection - Improved intrinsic tolerance of the myocardium to an imposed stress.

Catalase (CAT) - The antioxidant enzyme that catalyzes the conversion of hydrogen peroxide to H_2O and O_2.

Coronary Flow (CF) - The volume of blood flowing through the coronary vasculature per minute, normalized for the size of the heart in milliliters per minute per gram of heart wet weight.

Efficiency - The quotient of cardiac function (COxSP) and oxygen consumption.

Free Radicals - A highly reactive class of molecules, categorized by the presence of an unpaired electron in the outer orbital.

Glutathione (GSH) - A tri-peptide with a reduced sulfhydryl group, which is capable of being oxidized by glutathione peroxidase during the conversion of hydrogen peroxide to water.

Glutathione Peroxidase (GPX) - The anti oxidant enzyme that catalyzes the conversion of hydrogen peroxide to H_2O.

Heart Rate (HR) - The rate at which the heart contracts, in beats per minute.

Heat Shock Protein 72 (HSP72) - The 72 kilo Dalton inducible form of a protein in a class of proteins called stress proteins. Implicated in the facilitation of cardioprotective mechanisms. The abbreviation HSP70 represents both the constituiative and inducible form of the protein.

Ischemia- Period of no-flow in the myocardium during which the delivery of substrates and the removal of metabolic byproducts is prevented.

Isolated Heart Perfusion - Procedure for measuring the function of the heart in vitro by providing artificial blood flow to the heart and allowing the heart to pump independently from the organism.

Krebs-Henseleit Buffer - Solution used to perfuse the hearts containing essential ions and substrates at an osmolarity and pH similar to that observed in vivo.

Lactate Dehydrogenase (LDH) - The enzyme which catalyzes the reversible reaction from Lactate to Pyruvate. It is normally located in the cytosol of all cells and its presence in the coronary effluent indicates sarcolemma damage, which may lead to cellular necrosis.

Langendorff Perfusion - Isolated heart perfusion in which the heart does not perform any external work, as the heart only contracts against a retrograde perfusion pressure.

Lω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) - Competitive inhibitor of all three isoforms of nitric oxide synthase.

N-(2-Mercaptopropionyl)glycine (MPG) - Pharmacological, Potent free radical scavenger.

Myocardium - The heart muscle.

Nitric Oxide (NO) - A reactive nitrogen species produced by the action of nitric oxide synthase, which is responsible for signaling vasodilatation and initiating other cell-signaling events.

Nitric Oxide Synthase (NOS) - The enzyme that produces nitric oxide during the enzymatic conversion of L-arginine to L-citrulline. The three predominant isoforms include: eNOS, the endothelial isoform; iNOS, the inducible isoform; and nNOS the neuronal isoform.

Oxidative Stress - A stress in an organism caused by increased production of free radicals or reactive oxygen species.

Preconditioning - Any treatment capable of triggering a cardioprotective response in the myocardium that can protect against subsequent stresses. Preconditioning falls into two general categories, the early and late phase. The early phase appears minutes following a treatment, and is generally characterized by post-translational modification of existing proteins. The late phase occurs anywhere

from hours to days following treatment, and is generally characterized by de-novo synthesis of proteins.

Reactive Oxygen Species (ROS) - A class of highly reactive molecules, including free radicals, which can cause oxidative stress.

Superoxide Dismutase (SOD) - The anti oxidant enzyme that catalyzes the conversion of superoxide to hydrogen peroxide. The predominant isoforms include: MnSOD, located in the mitochondria; and CuZnSOD, located in the cytosol of the cell.

Systolic Pressure (SP) - The maximum pressure in the aorta during the contraction of the left ventricle, measured via the placement of a pressure transducer at the level of the aortic valve.

Uncoupling Protein 2 (UCP2) - The isoform of a protein, predominantly expressed in cardiac tissue, responsible for the uncoupling of mitochondrial respiration.

Working Heart Perfusion - Isolated heart perfusion in which the heart functions in a physiological, recirculating manner while producing measurable external work. Detailed in Figure 1.

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Vita

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